ANALYTICAL SCIENCES JANUARY 2016, VOL. 32

A Microfluidic Approach to Investigating a Synergistic Effect of Tobramycin and Sodium Dodecyl Sulfate on *Pseudomonas aeruginosa* Biofilms

Soojeong SHIN,* Ishtiaq AHMED, Jangsun HWANG,** Youngmin SEO,** Eunwon LEE,** Jonghoon CHOI,*,** Sangjun MOON,***† and Jong Wook HONG,*,**†**

**Department of Bionano Engineering, Hanyang University, Ansan Kyunggi-do 426-791, Korea*
***Department of Bionano Technology, Graduate School, Hanyang University, Seoul 133-791, Korea*
****Cybernetics Laboratory, Department of Robotics Engineering, DGIST, Daegu 711-873, Korea*

In recent years, a microfluidic technology has contributed a significant role in biological research, specifically for the study of biofilms. Bacterial biofilms are a source of infections and contamination in the environment due to an extra polymeric matrix. Inadequate uses of antibiotics make the bacterial biofilms antibiotic resistant. Therefore, it is important to determine the effective concentration of antibiotics in order to eliminate bacterial biofilms. The present microfluidic study was carried out to analyze the activities of tobramycin and sodium dodecyl sulfate (SDS) against *Pseudomonas aeruginosa* biofilms with a continuous flow in order to achieve a greater delivery of the agents. The results show that a co-treatment of tobramycin and SDS significantly reduced the biomass of biofilms (by more than 99%) after 24 h. Tobramycin and SDS killed and detached bacteria in the cores of biofilms. Evidently, our data suggest that a microchannel would be effective for both quantitative and qualitative evaluations in order to test combinatorial effect of drugs and chemicals on a complexed biological system including biofilm.

Keywords Microfluidic, *Pseudomonas aeruginosa*, sodium dodecyl sulfate, tobramycin, confocal laser scanning microscope

(Received August 31, 2015; Accepted October 30, 2015; Published January 10, 2016)

Introduction

Pseudomonas aeruginosa is known to be an opportunistic human pathogen, causing infections of the eyes, ear, skin, urethra and respiratory tract in cystic fibrosis (CF) and burn patients, as well as other immune-compromised individuals. Surface-attached bacterial communities, or biofilms, affect the antibiotics, biocides, and other antimicrobial agents a thousand times more than their planktonic populations.[1] Biofilm bacteria are much more resistant to antibiotic treatment, as well as to the host immune response; we are only beginning to understand the reasons for this biofilm resistance.[2-4] The latest findings have revealed that biofilms have significant roles in treating infectious disease, such as to act as a strong barrier against most of the in-practice medicines. This characteristic of biofilms makes them extremely difficult to control in medical and industrial settings. *P. aeruginosa* biofilms are thought to be linked with increased antibiotics resistance due to a lack of efficacy of the current therapies.[5,6]

Bacterial biofilms are among the complex organization of communal bacterial cells, which adhere to various biotic and abiotic surfaces (such as hydrophobic, hydrophilic, nanotopography, and charge). The biofilm matrix provides a protective shield to bacterial species that contributes ominously to several clinical and environmental challenges, including antibiotic resistance, symptomatic inflammation, recurrence, and the spread of infectious emboli, reported in previous studies.[7,8] The microbes in biofilm are protected from harsh conditions, such as shear stress, nutrient scarcity, pH fluctuations, oxygen radicals, disinfectants, and antibiotics. Extra polymeric substances engage antimicrobial agents, and minimize the physical stress in microbial communities.[9,10] Donlan[11] reported that rough surfaces support and enhance the biofilm formation opportunities due to the presence of beneficial local environments with reduced shear stress. Kinnari *et al.*[12] calculated that porous materials have advantages of biofilm formation compared with dense and smooth materials, because the attachment is affected by the degree of porosity and the permeability distribution.

Conventional formats for investigating biofilm formation have some obvious disadvantages of requiring large volumes, which is not suited for high-throughput analysis, and does not facilitate spatial and temporal control of biofilm formation.[13,14] The microfluidic devices present a promising platform for bacterial biofilms studies. They provide a closed system where bacterial biofilms can interact with hydrodynamic environments.[15] Stable fluid flows yield fast response times due to the low Reynolds number, to generate a gradient of the chemical attractant and to monitor bacterial chemotaxis.[16,17]

Here, we designed a microfluidic biofilm experiment system

† To whom correspondence should be addressed.
E-mail: nanobiomems@dgist.ac.kr (S. M.); jwh@hanyang.ac.kr (J. W. H.)

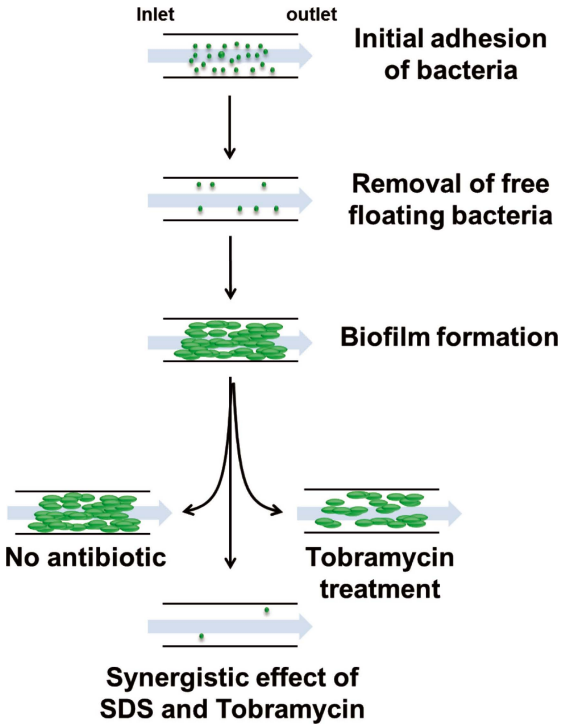

Fig. 1 Overall scheme of biofilm formation in a microfluidic device and an antibiotic treatment.

so as to determine the synergetic efficacy by a combinatorial chemical treatment for the eradication of *P. aeruginosa* biofilms attached to a surface (Fig. 1). Biofilm formation was induced in the microchannel, and the antibiotic susceptibility of the biofilm was examined. Moreover, SDS was added in the microchannel with tobramycin so as to observe a significant alteration of the biofilm, which may provide information about pertinent antibiotics and their quantity for high-throughput *P. aeruginosa* biofilm control. We studied how efficiently tobramycin and SDS penetrated and detached the established biofilms inside the microchannel using confocal imaging analysis.

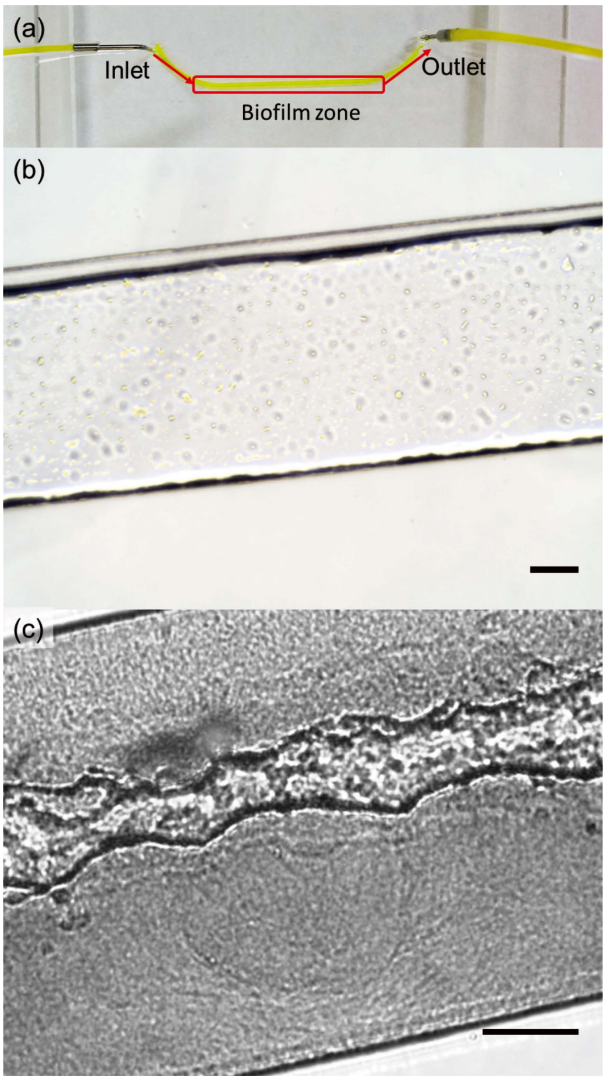

Fig. 2 Biofilms cultivated in a microfluidic device. (a) Design of the microchannel for observing *P. aeruginosa* biofilm. (b) Microscopic figure after seeding floated *P. aeruginosa*. (c) After a 72-h culture, biofilm was formed inside the microchannel. Scale bar, 100 µm.

Experimental

Strain and culture conditions

The *P. aeruginosa* KCTC 2004 strain was obtained from a Korean culture-type collection, and used in the current experiments. The bacterial colonies were grown on nutrient agar plates (Beef extract 3.0 g/L, Peptone 5.0 g/L, Bacto agar 5 g/L) at 37°C under an anaerobic condition (5% CO_2) for 24 h, and inoculated into a fresh LB medium (NaCl 10 g/L, Tryptone 10 g/L, Yeast extract 5.0 g/L) at 37°C for overnight. Overnight culture was used to inoculate fresh LB media to an initial OD_{600} of 0.05, and used as an inoculum in a hydrodynamic biofilms model. All chemicals were purchased from Sigma Aldrich (St. Louis, MO) and Becton, Dickinson and co. (Franklin Lakes, NJ).

Biofilm chip design and fabrication

The polydimethylsiloxane (PDMS) (Sylgard 184; Dow Corning Corp., USA) microchannel was fabricated using conventional soft lithographic techniques, as described previously.[18] In brief, a device is comprised of a bottom glass slide and a PDMS layer with a microchannel containing a single inlet for the introduction of bacterial seeds and fresh nutrient media and an outlet for the collection of waste. A mixture of PDMS pre-polymer and its curing agent (10:1 ratio) was poured over the mold and baked at 80°C for 35 min. The microscopic glass slide and PDMS replica were pretreated with oxygen plasma (oxygen flow rate of 10 sccm, 100 W, 30 s) in a plasma etcher (Femto Plasma system, Diener, Germany) before being bonded to each other.[19] The dimensions of microchannel were 2.0 cm long, 67 µm deep, 500 µm wide (Fig. 2(a)). The flow rate was controlled by fusion 100 touch dual-injection syringe pumps (Revodix Inc., Korea). To maintain the humidity, moist air was flowed under a temperature-controlled chamber so as to control the temperature of the device at 37°C.

Antibiotic susceptibility of planktonic bacteria

The inhibitory concentration of planktonic bacteria was investigated with exponentially grown *P. aeruginosa*. An antibiotic susceptibility assay was performed with the standard two-fold dilution method, similar to the Clinical and Laboratory Standards Institute guidelines.[20] Tobramycin stock solutions

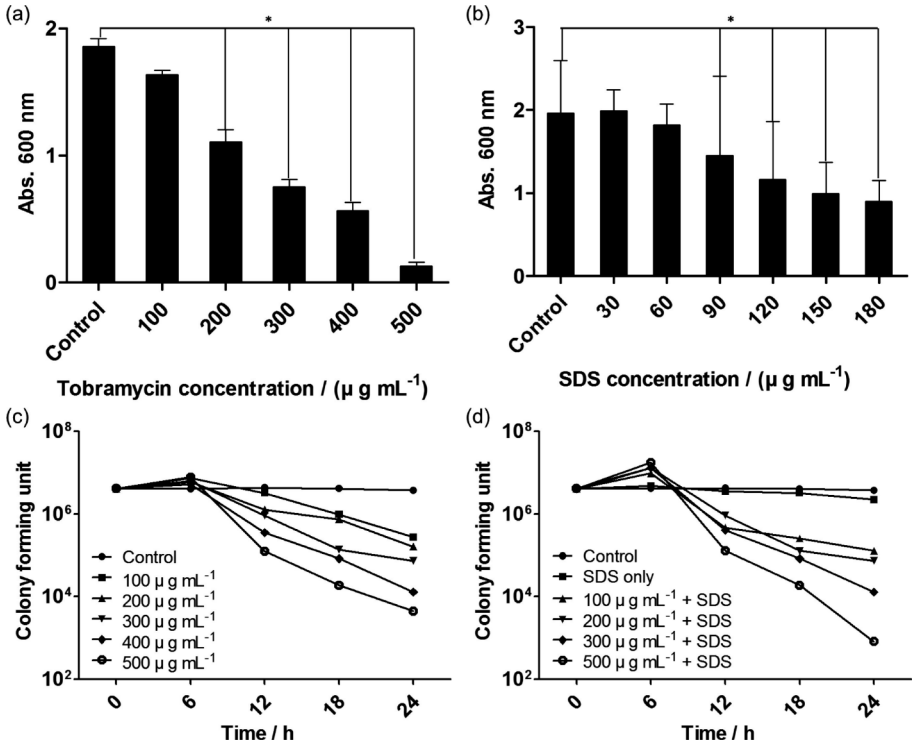

Fig. 3 Antibiotic and SDS susceptibility. (a) The viability of *P. aeruginosa* was quantified under different concentrations (0 – 500 μg mL^{-1}) of tobramycin by a spectrophotometer. The error bars indicate the standard deviation of three measurements at 600 nm. (b) SDS decreases some viability of *P. aeruginosa* under a non-hazardous concentration (180 μg mL^{-1}) for humans. (c) and (d) represent colony forming unit per 1 mL of suspension after treatment of (c) tobramycin and (d) combination of tobramycin and SDS. Asterisk (∗) denoted significance with control group ($p < 0.05$).

were prepared less than 24 h before use at the required concentration. To determine the inhibitory concentration by time, the bacterial cells were incubated for 24 h in an LB medium with the addition of different concentrations of antibiotics and SDS, and then remained as floating cells and detached biofilm by trypsin. Some of the cell suspension was analyzed spectrophotometically so as to count the cell number. The remaining cell suspension was re-cultured in an agar plate for counting the colony forming units of each experimental set.

Microfluidic device biofilm formation and evaluation

Biofilms were cultivated in a microfluidic device at 37°C, as described Dai *et al.*[21] Before introducing *P. aeruginosa*, the microfluidic device was calibrated using a phosphate buffer saline (pH 7.2) with a flow rate 0.05 mL min^{-1}, followed by autoclaved LB media for 4 h in order to remove any bubbles and impurities present in the channel. Bacterial cells were introduced with 1 mL Gastight syringes from the inlet at a flow rate of 0.025 mL min^{-1} (Fig. 2(b)). After 1 min, the syringe pump was stopped for 1 h so as to allow the bacteria to adhere onto the glass substratum of the microfluidic device. We washed the microchannel with sterilized phosphate buffer saline to eliminate the floating and weakly bonded cells at a flow rate of 0.5 mL min^{-1}. After the adhesion time, the flow of nutrient media was resumed at a flow rate of 0.015 mL min^{-1}. After 72 h, when mature biofilms were formed (Fig. 2(c)), the antibiotics were introduced at 0.010 mL min^{-1} a flow rate from the inlet.

Confocal laser scanning microscopy of biofilms

A Zeiss LSM 510 META equipped with a water-immersion of 63 objective (Carl Zeiss, Jena, Germany) was used to observe the bacterial cell activities in the microfluidic device. Briefly, the live and dead cells distribution in biofilms was assessed by staining for 15 min with 100 μL LIVE/DEAD *Bac*Light Bacterial Viability Kit (Invitrogen Corp., NY, USA). A *Bac*Light stock solution was prepared instantly before use. The final concentrations of both components (Syto-9 and propidium iodide) were diluted at a 1:300 ratio in a NaCl solution containing 5% dimethyl sulfoxide (DMSO), respectively. The green (SYTO 9) was excited at 488-nm and detected with a 540/75-nm band pass emission filter, respectively.[22]

Image and data analysis

The CLSM images were evaluated for quantification of the structural elements (biomass, average thickness, roughness coefficient and substratum coverage) of biofilms by image-processing software, COMSTAT, designed by Heydorn *et al.*[23] In brief detail, images were attained at a distance of 10 – 15 mm from the inlet of the flow channel. Images were attained at 1 ± 0 to 2 ± 0 μm intervals, and therefore the number of images varied according to biofilm thickness in each stack of images. The program COMSTAT was written as a script in MATLAB 5.1 (The MathWorks, MA, USA), equipped with the Image Processing Toolbox. Before quantification, each image stack was thresholded, and the image information included the name and number of images in each stack, the threshold value, the filtering method, x (pixel size), y (pixel size) and z (pixel size). The quantification results were saved in a report file.

Results and Discussion

In the present study, we investigated a synergistic effect of SDS and tobramycin in microchannel formed biofilms, which provide an on line monitoring facility. *P. aeruginosa* forms flat and uniform biofilms in microfluidic devices with continuous flow of the nutrient medium. Biofilms provide protection to members of biofilm community, and hence biofilms were exposed to the antibiotic tobramycin by using a microfluidic assay, as described earlier.

Before a microchannel experiment, the biofilm formation and evaluation of inhibition concentration for tobramycin and SDS was observed in cell culture dishes (Fig. 3). Most antibacterial candidates were decreased by the planktonic bacteria viability, but were not effective under biofilms conditions. However, tobramycin showed the biofilm inhibition effect due to a decrement of the cell numbers. Oppositely, the results of SDS exposure to *P. aeruginosa* planktonic cells revealed that SDS has some cell-number decrement activity. We choose a maximum of 180 μg/mL SDS, the usually used concentration in different interleukin injections, as a solubilizing agent. This means that this amount has no substantial adverse effect on the human body. In the 24 h biofilm inhibition test (Figs. 3(c) and 3(d)), both graphs showed increments of the colony-forming unit after 6 h, except for the control and SDS experimental sets, which would be an indirect representation that tobramycin did not give any sudden effect on the biofilm. However, as time went on, the number of live cells decreased, and the combinatorial treatment of tobramycin and SDS showed a more adverse effect on the biofilm.

As a model of *P. aeruginosa* biofilm formation and inhibition by tobramycin and SDS, a PDMS-based microchannel was applied. Since the PDMS device has a hydrophobic surface, biofilm started to form from the bottom-slide glass, which made it easy to observe the 2-dimensional biofilm conformation. After 72 h of maturation of 2-D biofilms grown in the microchannel, similar to the culture condition of cell culture dishes, tobramycin was introduced for 24 h of continuous flow. Green fluorescent Cyto 9 was used to stain live cells, as well as a partial amount of dead cells aggregated inside the biofilm. Thus, the unstained area in the analyzed image of Fig. 3 represented biofilm eradication compare to the control sample. As an increment of the tobramycin concentration, *P. aeruginosa* biofilm showed a significant effect on biofilm eradication (99%). However, this combination was unable to eradicate all of the biofilm community attached onto the glass substratum in the microfluidic device (Fig. 4(a)). We introduced 180 μg/mL of SDS additionally (Fig. 4(b)), and matured biofilms were eradicated faster. The SDS treatment only left 60% of the biofilm associated cells, while the combination of SDS and tobramycin removed more than 99% of biofilms embedded cells in the continuous flow as well as most of the glass-attached cells. Therefore, the combination of SDS and tobramycin was more effective than the SDS or tobramycin alone.

The synergistic effect of SDS and tobramycin was investigated for a detailed characterization of various biofilm factors, such as the number of colonies at the substratum, normalized biomass amount, average thickness of biofilm, substratum coverage, roughness coefficient, and the surface-to-volume ratio. In all factors, the combination of SDS with tobramycin improved the eradication of biofilm. More concentrated tobramycin and SDS was treated, and the biofilm thickness and cell numbers were reduced. Especially, the roughness and the surface-to-volume ratio of the biofilm increased, which may represent the detachment of biofilm-associated bacteria randomly and losing cell–cell connections in the biofilm. By increasing the surface-to-volume ratio, an acceleration of the tobramycin and SDS effect is expected.

Tobramycin and SDS disrupted the biofilm structure quickly, so that the cells eventually washed out with the continuous flow, but their detailed function would be different. Tobramycin reduced both the cell numbers and the colony forming unit (Figs. 3(a) and 3(c)), but its activity was not so efficient to

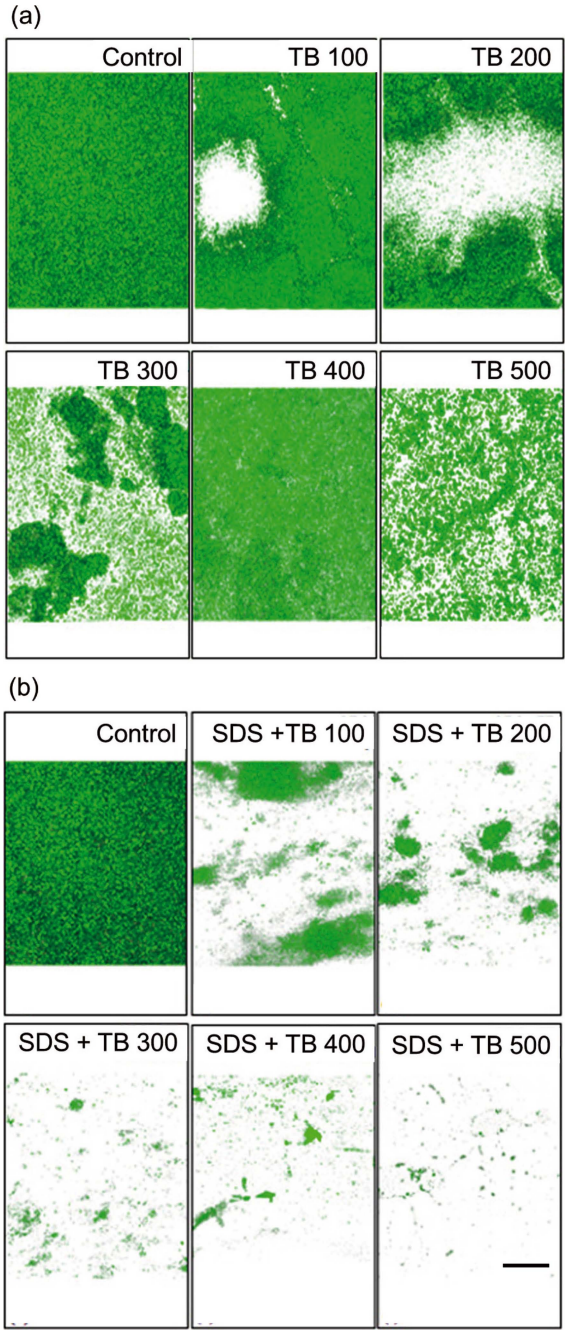

Fig. 4 Confocal laser scanning microscopy of *P. aeruginosa* biofilms grew in a characteristic pattern with a lawn of bacterial growth on the glass substratum and the effects of tobramycin and SDS. (a) Increment of the antibiotic concentration induced more loss of bacterial viability. (b) Combinatorial effect of 180 μg mL^{-1} of SDS and tobramycin resulted incredible inhibition of biofilms growth even attached on glass slide. Scale bar = 50 μm.

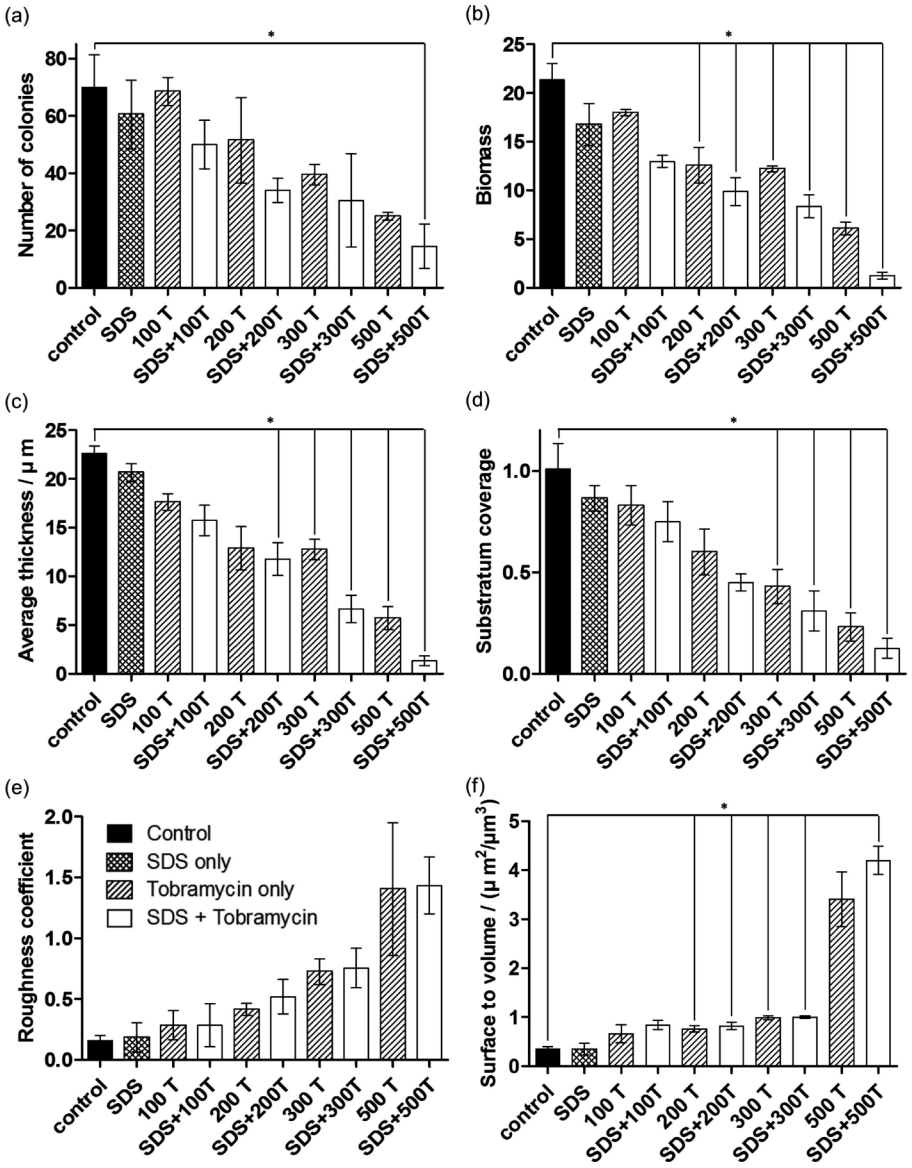

Fig. 5 Characteristics of *P. aeruginosa* biofilms after a 24-h treatment with SDS and tobramycin under different concentrations were evaluated by a COMSTAT analysis of confocal images. (a) Number of colonies at substratum (b) normalized biomass amount (c) average thickness of biofilm (d) substratum coverage (e) roughness coefficient and (f) surface to volume ratio were analyzed. Asterisk (∗) denoted significance with control group ($p < 0.05$).

bacterial tightly attached onto the glass slide. (Fig. 4(a)). On the other hand, SDS-only treated results also kill bacterial, but the percentage was relatively low (40% in Fig. 3(d)). Instead, SDS showed great activity on bacterial detachment on biofilm (Fig. 4(b)). Thus, a combinatorial treatment resulted in more than 99% of surface cleaning in the glass slide. Therefore, a co-treatment of tobramycin and SDS gives not only a total concentration increment, but also synergetic effect on the eradication of biofilm by both bacteria killing and detachment from the surface.

Biofilm bacteria susceptibility testing revealed that inhibitory concentrations were significantly higher in biofilm-embedded *versus* planktonic bacteria for all reported antimicrobials. The inhibitory concentration of the tobramycin was significantly effective to eradicate *P. aeruginosa* biofilms in the presence of SDS. From several solubilizing and antibacterial agents, SDS combined with tobramycin have stronger activity against *P. aeruginosa*. A similar approach was carried out for the combination of bismuth and lipophilic thiol EDT, and even more stronger when combining tobramycin with Bi-EDT.[24-26] However, the antimicrobial action of SDS is not well understood.

Chen and Stewart[27,28] used various chemicals for the removal of mixed *P. aeruginosa-Klebsiella pneumoniae* biofilms. They reported a 49% reduction in cell counts with the dispersal of biofilm cells after an EDTA treatment (10 mM). Viscosity of *P. aeruginosa-Klebsiella pneumoniae* biofilms was also determined by adding several cations. They reported that SDS increased the viscosity of biofilms, so authors concluded that electrostatic interactions play important roles in biofilm cohesion, and that SDS are compelling cross-linkers of biofilm. Turakhia and Characklis[29] reported that *P. aeruginosa* biofilms cohesiveness increased after calcium addition in the growth media, which resulted in a decreased detachment of cells. While EGTA mixed with sewage sludge speeded up the cells

detachment from biofilms, the killing rate was fivefold lower than EDTA. No found significant difference was under different flow (laminar and turbulent) regimes upon susceptibility testing, but during biofilm formation the flow conditions played an important role to construct the biofilm structure, but did not affect the SDS activity of controlling the biofilms.[30,31] The effects of SDS on the biofilm viability, eradication, and recovery create several issues concerning the probable influence of chemical agents and doses as well as the pervasiveness of resistance and cross-resistance.[32,33] The dispersal process and the increased cell permeability enabled by SDS revealed the cells detachment and killing after combined SDS and tobramycin treatments.

P. aeruginosa causes pulmonary infections, such as cystic fibroses (CF) and bronchiectasis. These infections are also associated with endotracheal tubes.[34,35] The protection of bacterial cells embedded in a biofilm matrix from antimicrobial agents is considered to be extrapolymeric substances that provide physical protection to bacterial cells, and thus antibiotics may not be able to penetrate and exert their effect. Usually, the recommendations were carried out based on planktonic cultures experiments. Simoes *et al.*[36] investigated whether such results do not minimize the growth conditions found on the surface, where biofilms are formed. In recent years, new approaches of therapy and methods of *P. aeruginosa* infection in CF patients have been explored. Only small numbers of antibiotics, natural or synthetic compounds, have been investigated concerning the removal of *P. aeruginosa* biofilms. Multi-drug resistance and tolerance of *P. aeruginosa* has led to new interests in developing new strategies that restrict its adherence at the surface, and stop communications with other pathogens during biofilm formation, as reported by Ni *et al.*[37] In the same manner, we treated both tobramycin and SDS for *P. aeruginosa* biofilm eradication; a combinatorial treatment revealed that more than 99% of biofilm was removed. Its synergetic effect would be caused by a different function of its chemicals; tobramycin kills bacteria, whereas SDS induces cell detachment and junction loosening in biofilm.

Especially, we grew biofilm and performed inhibition tests in microchannels. In previous studies,[38-40] the development of microfluidic systems considered to be effective tools of biological assays, that can generate a linear concentration gradient. We fabricated a simple microchannel to monitor biofilms eradication after exposure of specific chemicals. The proposed assay can be helpful for *in situ* analysis by promising an *in vivo* environment, such as that in blood vessels.

Conclusions

We demonstrated an *in situ* bacterial biofilm formation and inhibition by a tobramycin and sodium dodecyl surface (SDS) susceptibility comparison. The results of the microchannel experiment showed quantitative insight concerning the roles of tobramycine and SDS. Moreover, 2D formed biofilm in a microchannel made it possible to perform qualitative analysis of a biofilm by the COMSTAT program. The results imply that the combination of tobramycin and SDS significantly eradicates the *P. aeruginosa* biofilms by killing and detaching cells from the biofilm, respectively. This microchannel system can be applied for the understanding of the segregated structure of biofilm. Further investigation is needed to apply these findings clinically. Also, detailed biochemical and genetic studies are required for understanding the precise mechanism of the antibiotic susceptibility of biofilms.

Acknowledgements

This work was supported by the research fund of Hanyang University (HY-2013-201300000002248).

References

1. N. Høiby, T. Bjarnsholt, M. Givskov, S. Molin, and O. Ciofu, *Int. J. Antimicrob. Agents*, **2010**, *35*, 322.
2. T. F. Mah, B. Pitts, B. Pellock, G. C. Walker, P. S. Stewart, and G. A. O'Toole, *Nature*, **2003**, *426*, 306.
3. S. M. Moskowitz, J. M. Foster, J. Emerson, and J. L. Burns, *J. Clin. Microbiol.*, **2004**, *42*, 1915.
4. H. Hirakawa and H. Tomita, *Front. Microbiol.*, **2013**, *4*, 114.
5. N. Hoiby, B. Frederiksen, and T. Pressler, *J. Cystic Fibrosis*, **2005**, *4(Suppl. 2)*, 49.
6. R. M. Donlan and J. W. Costerton, *Clin. Microbiol. Rev.*, **2002**, *15*, 167.
7. A. A. Prince, J. D. Steiger, A. N. Khalid, L. Dogrhamji, C. Reger, S. Eau Claire, A. G. Chiu, D. W. Kennedy, J. N. Palmer, and N. A. Cohen, *Am. J. Rhinol.*, **2008**, *22*, 239.
8. E. Deligianni, S. Pattison, D. Berrar, N. Ternan, R. Haylock, J. Moore, S. Elborn, and J. Dooley, *BMC Microbiol.*, **2010**, *10*, 38.
9. V. Gupta, *Expert Opin. Invest. Drugs*, **2008**, *17*, 131.
10. D. W. Sims, "*Advances in Marine Biology*", **2007**, Academic Press, Waltham.
11. R. M. Donlan, *Emerg. Infect. Dis.*, **2002**, *8*, 881.
12. T. J. Kinnari, J. Esteban, N. Z. Martin-de-Hijas, O. Sanchez-Munoz, S. Sanchez-Salcedo, M. Colilla, M. Vallet-Regi, and E. Gomez-Barrena, *J. Med. Microbiol.*, **2009**, *58*, 132.
13. C. Sternberg and T. Tolker-Nielsen, "*Current Protocols in Microbiology*", ed. M. Grigg, A. McBride, J. M. Quarles, B. Stevenson, and R. K. Taylor, **2005**, Chap. 1, Sect. B2, John Wiley & Sons, Incorporated, Hoboken.
14. M. R. Benoit, C. G. Conant, C. Ionescu-Zanetti, M. Schwartz, and A. Matin, *Appl. Environ. Microbiol.*, **2010**, *76*, 4136.
15. V. Janakiraman, D. Englert, A. Jayaraman, and H. Baskaran, *Ann. Biomed. Eng.*, **2009**, *37*, 1206.
16. T. M. Keenan and A. Folch, *Lab Chip*, **2008**, *8*, 34.
17. T. Ahmed, T. S. Shimizu, and R. Stocker, *Integr. Biol.*, **2010**, *2*, 604.
18. Y. Meng, Y. Li, C. D. Galvani, G. Hao, J. N. Turner, T. J. Burr, and H. C. Hoch, *J. Bacteriol.*, **2005**, *187*, 5560.
19. M. K. Chaudhury and G. M. Whitesides, *Langmuir*, **1991**, *7*, 1013.
20. M. E. Olson, H. Ceri, D. W. Morck, A. G. Buret, and R. R. Read, *Can. J. Vet. Res.*, **2002**, *66*, 86.
21. J. Dai, S. H. Yoon, H. Y. Sim, Y. S. Yang, T. K. Oh, J. F. Kim, and J. W. Hong, *Anal. Chem.*, **2013**, *85*, 5892.
22. M. Musken, S. Di Fiore, U. Romling, and S. Haussler, *Nat. Protocols*, **2010**, *5*, 1460.
23. A. Heydorn, A. T. Nielsen, M. Hentzer, C. Sternberg, M. Givskov, B. K. Ersboll, and S. Molin, *Microbiology*, **2000**, *146*, 2395.
24. C. L. Wu, P. Domenico, D. J. Hassett, T. J. Beveridge, A. R. Hauser, and J. A. Kazzaz, *Am. J. Respir. Cell Mol. Biol.*, **2002**, *26*, 731.
25. P. Domenico, L. Baldassarri, P. E. Schoch, K. Kaehler, M. Sasatsu, and B. A. Cunha, *Antimicrob. Agents Chemother.*, **2001**, *45*, 1417.

26. M. Halwani, S. Blomme, Z. E. Suntres, M. Alipour, A. O. Azghani, A. Kumar, and A. Omri, *Int. J. Pharm.*, **2008**, *358*, 278.
27. X. Chen and P. S. Stewart, *Water Res.*, **2000**, *34*, 4229.
28. X. Chen and P. S. Stewart, *Appl. Microbiol. Biotechnol.*, **2002**, *59*, 718.
29. M. H. Turakhia and W. G. Characklis, *Biotechnol. Bioeng.*, **1989**, *33*, 406.
30. M. Simoes, M. O. Pereira, S. Sillankorva, J. Azeredo, and M. J. Vieira, *Biofouling*, **2007**, *23*, 249.
31. M. Simoes, L. C. Simoes, M. O. Pereira, and M. J. Vieira, *Biofouling*, **2008**, *24*, 35.
32. D. Davies, *Nat. Rev. Drug Discovery*, **2003**, *2*, 114.
33. P. Gilbert and A. J. McBain, *Clin. Microbiol. Rev.*, **2003**, *16*, 189.
34. S. Cai, J. Zhang, and G. Qian, *Zhonghua Jiehe He Huxi Zazhi*, **2001**, *24*, 339.
35. A. S. Prince, *N. Engl. J. Med.*, **2002**, *347*, 1110.
36. M. Simoes, M. O. Pereira, and M. J. Vieira, *Water Res.*, **2005**, *39*, 5142.
37. N. Ni, M. Li, J. Wang, and B. Wang, *Med. Res. Rev.,* **2009**, *29*, 65.
38. N. Li Jeon, H. Baskaran, S. K. Dertinger, G. M. Whitesides, L. Van de Water, and M. Toner, *Nat. Biotechnol.*, **2002**, *20*, 826.
39. P. J. Hung, P. J. Lee, P. Sabounchi, R. Lin, and L. P. Lee, *Biotechnol. Bioeng.*, **2005**, *89*, 1.
40. J. Diao, L. Young, S. Kim, E. A. Fogarty, S. M. Heilman, P. Zhou, M. L. Shuler, M. Wu, and M. P. DeLisa, *Lab Chip*, **2006**, *6*, 381.