The Effect of Nondialyzable Material (NDM) Cranberry Extract on Formation of Contact Lens Biofilm by *Staphylococcus epidermidis*

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Purpose. To assess the effects of NDM from cranberries on *Staphylococcus epidermidis* biofilm formed on soft contact lenses.

Methods. Soft contact lenses were incubated in Tryptic Soy Broth (TSB) together with *S. epidermidis* (ATCC35984/RP62A) and various concentrations of NDM, and inspected by scanning electron and confocal microscopy. The TSB was collected after sonification and monitored turbidometrically.

Results. NDM at ≥500 μg/mL concentration caused a significant ($P < 0.01$) reduction of biofilm. Scanning electron microscopy of biofilm in the presence of 500 to 1000 μg/mL NDM confirmed these results. In control lenses, multilayered mushroom-shaped biofilm and complete coverage of the lens surface were seen, whereas after incubation with 500 μg NDM per mL TSB, the biofilm was thinner with smaller protuberances, and exposed lens surface was partially seen. In samples incubated with 1000 μg NDM per mL TSB, the lens surface was clearly seen between sporadic microcolonies.

Conclusions. NDM reduces formation of biofilm on soft contact lenses. This has important implications for the prevention of contact lens–related corneal infections caused by *S. epidermidis*. (*Invest Ophthalmol Vis Sci.* 2011;52:4929–4934) DOI:10.1167/iovs.10-5335

Bacterial contamination of contact lenses can lead to the development of corneal infections, including sight-threatening microbial keratitis. In a recent review[1] of medical cases of bacterial keratitis treated in an eye center in Australia, over one-third of the cases were attributed to contact lens wear. The infections were caused by Gram-positive bacteria in 27% of cases, Gram-negative bacteria in 11%, and multiple microbes in 13.8%. McLaughlin-Borlace et al.[2] provided evidence of the presence of biofilm on the surfaces of contact lens samples collected from patients with a clinical diagnosis of microbial keratitis.

Biofilms have also been shown to develop on contact lens storage cases.[2,3] Indeed, storage cases are sometimes the primary source of contamination. In a study of patients with corneal infections, bacterial biofilms were found in 17 of 20 storage cases examined.[2] The latter rate was found to be higher than the rate of biofilm formation on the contact lenses themselves. Additionally, the identical organism was isolated from the lens case and the infected corneal ulcers in 9 of 19 samples.[4]

Although antibiotic therapy and activated host defenses can kill biofilm-derived planktonic cells, extreme antibiotic dosages over a long period are needed to kill biofilm bacterial cells.[5,6] This antibiotic resistance has been attributed to the restricted penetration of antimicrobials into the biofilms and the metabolic inactivity of starved bacteria in the deep biofilm layers because of the limited diffusion of nutrients.[4,6] Specific genes may be expressed more in biofilms that help protect the bacterium from antibiotics, such as *ndvB* in *Pseudomonas aeruginosa.*[7]

Studies aimed at overcoming biofilm bacterial resistance have shown that interference with biofilm formation may prevent the development of serious infections.[5,8–10] Several methods have yielded promising results, including the application of anti-adhesion compounds and detachment of bacteria from biological surfaces, such as oral mucosa.[11–13] The aim of our study was to determine whether material extracted from cranberries, termed nondialyzable material (NDM), which is known to inhibit bacterial adherence,[12] can inhibit staphylococcal biofilm formation on soft contact lenses.

Materials and Methods

A slime-producing strain of *Staphylococcus epidermidis* (ATCC35984/RP62A)[14] was provided by Wilma Ziebuhr from the Institute for Molecular Infections–Biology, Würzburg, Germany. Silicone-hydrogel contact lenses containing 42% polymer (Filcon 1B) and 58% water (Ocular Sciences, Concord, CA), with a diameter of 14.2 mm, were purchased from Hanita Lenses Company (Kibbutz Hanita, Israel). The contact lenses were removed from their package and washed once with cold sterile phosphate buffered saline (PBS) before use. NDM was obtained as described previously[15] from concentrated cranberry juice made from the American cranberry, *Vaccinium macrocarpon*, and provided by Ocean Spray Cranberries (Lakeville-Middleboro, MA). Briefly, the concentrated juice was dialyzed at room temperature against distilled water in a 12,000 MW cutoff dialysis bags and lyophilized. NDM exhibits tannin-like properties: It is soluble in water, devoid of proteins, carbohydrates, and fatty acids, and was found to be rich in phenolic compounds (e.g., proanthocyanidins).[16,17] Further analysis performed by Ocean Spray revealed that this fraction is devoid of sugars, acids, and nitrogen and contains 0.35% anthocyanins (0.055% cyanidin-3-galactoside, 0.003% cyanidin-3-glucoside, 0.069% cyanidin-

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3-arabinoside, 0.116% peonidin-3-galactoside, 0.016% peonidin-3-glucoside, and 0.086% peonidin-3-arabinoside) and 65.1% proanthocyanidins.[18,19] NDM is soluble up to 4 mg/mL. At this concentration it lacks any detectable effect on viability as determined by colony counts method. Thus its minimal inhibitory concentration (MIC) is actually greater than 4 mg/mL, and because at higher concentrations it precipitates in aqueous solutions, its exact MIC cannot be determined.[17]

The NDM powder was dissolved in double-distilled water at a concentration of 4 mg per 1 mL, divided into aliquots, and maintained at a temperature of −20°C until use.

A single colony of *S. epidermidis* grown on 5% sheep blood–supplemented TSA was inoculated into 15 mL Difco TSB, containing glucose at 2.5 g/L. After 18 to 20 hours of overnight incubation at 37°C, the organisms were pelleted by centrifugation at 3000 rpm for 5 minutes and washed twice with 25 mL PBS 0.2 mM (pH = 7.5). The final bacterial pellet was resuspended in TSB to an optical density (OD) of 0.05 at 600 nm and then incubated for 4 hours at 37°C to obtain a logarithmic phase bacterial culture, which served to inoculate the experimental wells as described below. The OD evaluation was performed with a spectrophotometer (WPA CO800; Isogen Life Science, De Meern, the Netherlands).

Growth and Quantification of Biofilm on Contact Lenses

Contact lenses were placed in a 24-well plate, one contact lens per well. Two milliliters of TSB containing various concentrations of NDM in serial twofold dilutions, ranging from 2000 μg/mL to 62.5 μg/mL, were added to each well. This was followed by the addition of 100 μL of bacterial culture. Controls included TSB alone (TSB). In addition, wells containing contact lenses with TSB but no added bacteria were added to the controls to ensure lens sterility. After overnight incubation at 37°C each contact lens was carefully removed with sterile forceps, washed gently by dipping in a sterile PBS container, and placed in a sterile tube containing 1 mL TSB. To quantify the bacteria in the biofilm by spectrophotometry, and because conventional dyeing methods resulted in nonspecific dyeing of the lens surface itself (data not shown), tubes were placed in an ultrasonic bath (D80H; MRC Ltd, Holon, Israel) in cold water at 43 kHz. Two rounds of sonication and manual scraping of the lenses with a sterile spatula were performed to ensure removal of most of the biofilm bacteria from the lenses. Thereafter, the TSB containing bacteria was collected, and the OD was determined at 600 nm, using sterile TSB as a blank.

Scanning Electron Microscopy Evaluation of Contact Lens Samples

Round pieces were punched out of each contact lens using a sterile puncher to yield flat small circles approximately 5 mm in diameter. Each piece was washed with PBS and placed in a compartmentalized slide (Lab-Tek II chamber slide system, No. 154,534; Thermo Fisher Scientific, Inc., Waltham, MA). Lenses were then treated as described above, with adjustment to 0.7 mL fluid per well. A solution containing the desired treatment was added to each well, with the addition of a bacterial culture. The slides were incubated overnight at 37°C. Samples were subsequently washed carefully with PBS and fixed in 2% glutaraldehyde solution overnight at room temperature. Finally, the samples were transferred to the electron microscopy unit, where they were dehydrated, coated with gold by a low vacuum sputter coating, and scanned by scanning electron microscopy.

Confocal Microscopy Evaluation of Contact Lens Samples

Round pieces, 5 mm in diameter, were punched out of the contact lenses, washed with PBS, placed in a compartmentalized slide, and treated as described above. After overnight incubation, the samples were dyed with a bacterial viability kit Live/Dead BacLight, L-13,152; Invitrogen Ltd., Paisley, UK), a rapid epifluorescence staining method. The kit utilizes Syto 9 green and propidium iodide red fluorescent nucleic acid stains.[20] The dye was prepared according to the manufacturer's specifications. The samples were carefully decanted of fluid, and 15 mL of the dye solution was dripped onto the lens pieces. The slides were incubated in the dark for 15 minutes and then evaluated by a confocal microscope. As a final step, the slides were photographed with a Carl Zeiss laser scanning system (ILSM 510 Meta; Carl Zeiss, MicroImaging GmbH, Jena, Germany) at excitation levels of 488 nm for Syto 9, which stains live bacteria, and 543 mm for propidium iodide, which stains dead bacteria. Images were acquired at 2.1 μm intervals (Z-stacks; Zeiss LSM Image Browser; Carl Zeiss).[21] Postacquisition images were then processed (Zeiss LSM Image Browser and Matrox Inspector; Carl Zeiss). A histogram of each Z-stack profile was created (Matrox Inspector software; Carl Zeiss), and the area of green and red pixels was calculated by the program. To reduce noise, background levels were determined by evaluating areas where no bacteria were present. The background (30 gray levels) was then subtracted from all images. When we determined the gray level (an arbitrary measure of opacity) of blank lenses, we got a value of zero (data not shown) gray levels. Blank lenses were inappropriate control, because they do not have the same background fluorescence as the bacteria-containing lenses.

Statistical Analysis

Statistical analysis was performed with a commercially available statistical package (SPSS; SPSS, Chicago, IL). For each experiment, a minimum of four repetitions were performed for each concentration. The repetitions were considered to be statistically significant by Student's *t*-test when the *P* value was < 0.05. The significance of the difference between each sample and all the other treated samples and the control was determined with Student's *t*-test; a *P* value of 0.05 or less was considered statistically significant.

Results

The presence of NDM during bacterial growth caused a significant ($P < 0.01$) reduction of biofilm after 24 hours growth at ≥500 μg/mL (Fig. 1). Although both 500 and 1000 μg/mL NDM significantly reduced biofilm formation, there was no statistical difference between the 500 and 1000 values. In separate experiments (data not shown) biofilm was allowed to form, and then exposed to 250 and 500 μg/mL NDM. NDM showed no effect on detaching bacteria from preformed bio-

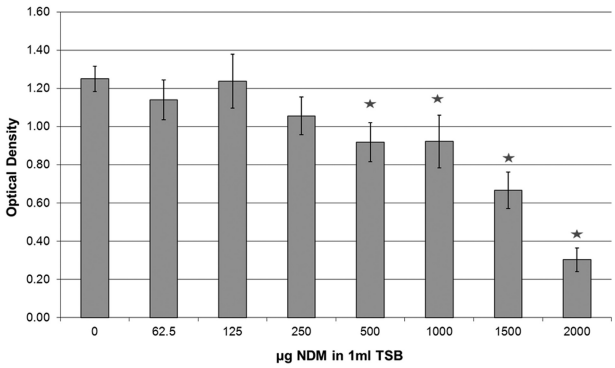


Figure 1. Minimal effective concentration of NDM in biofilms formed on contact lenses. Correlation between different concentrations of NDM and the concentration of adherent-biofilm bacteria, estimated by OD, after sonication in TSB. At 500 μg NDM per 1 mL TSB a trend starts to show: inhibition of biofilm growth as the concentration increases. Results are represented ±SEM. A minimum of four repetitions were performed for each concentration. ★Significant ($P < 0.01$) difference from culture devoid of NDM.

film. It should be stressed that at the concentrations used, NDM did not affect the viability of the bacteria.

Electron microscopy of biofilm formation on contact lenses in the presence of 500 and 1000 μg/mL NDM confirmed the results estimated by optical density (Fig. 2). In the control lenses, a multilayered mushroom-shaped biofilm is clearly seen, covering the surface with several layers of bacteria (Fig. 2a). In the lenses incubated with 500 μg NDM per ml TSB, the biofilm was thinner, with parts of the lens surface left exposed (Fig. 2b). In samples incubated with 1000 μg NDM per ml TSB, the lens surface was clearly seen between sporadic microcolonies (Fig. 2c).

Confocal Microscopy Findings

To estimate the fraction of dead versus live bacteria, biofilms were stained with a bacterial viability kit (Live/Dead BacLight), which utilizes Syto 9 to stain live bacteria in fluorescent green stain, and propidium iodide, which penetrates only damaged membranes and therefore stains only dead bacteria red.

Figure 3 shows three z-series, of two NDM treatments and a control. In each sample, photographs were taken starting at the lens plane surface and ending at the highest level of the biofilm that could be detected. The control (Fig. 3a) consisted of a thick layer of biofilm. The lower layers (stained red) were dead, possibly because of the excess of waste materials in the deeper layers of the biofilm. The biofilm treated with 500 μg/mL NDM (Fig. 3b) was deeper than the control but had almost no dead bacteria. In addition, it comprised mostly mushroom-shaped protrusions that were higher but not as substantial as the protrusions in the control. The biofilm treated with 1000 μg/mL NDM (Fig. 3c) was as deep as 500 μg/mL NDM and also had hardly any dead bacteria. It also comprised mostly mushroom-shaped protrusions. However, at this concentration, we noted that it was harder to find fields in which the protrusions were evident, making this concentration the most potent for use.

Computer analysis was performed for every fifth section of the same stack, starting at the lens surface. The amount of bacteria in each section was summed, and the bacterial concentration was analyzed in relation to the depth of the biofilm. The results are shown in Figure 3. A cross section extracted from the z-stack series in Figure 3 (x-z view of the sample) provided a clear image of the depth of the biofilm, the layering of bacteria, and the bacterial condensation. In each sample the 32nd z-plane (Fig. 4a) was chosen as representative of the midsample, and a cross section (Fig. 4b) was made at the same location (Zeiss LSM). In the TSB control (left, Fig. 4), dead bacteria were lined up underneath the live ones in a thick and regular layer of biofilm, with a mushroom-shaped protrusion visible on the top left of the section. In both NDM-treated samples (Fig. 4, right), only the protrusions were visible, with no underlying layer of dead bacteria. A histogram (Fig. 4c) of the red and green pixels in the above cross section was created (Matrox Inspector) to obtain a clearer view of the overall relationships of the live and dead bacteria. The threshold (blue line) was set at a gray level of 30 by sampling the line profiles of several cross sections. The pixel percentage of the cross section at 30 to 255 gray level was determined by the same program and plotted (Fig. 4d). We found that the scales of the histograms differed from one plot to the other. The overall amount of live bacteria was greater in the control than in the NDM-treated samples, possibly as a result of the NDM inhibition of bacterial adhesion. The control had a considerably large amount of dead bacteria as well. This finding was further clarified by plotting the percentage of coverage, indicating that the control biofilm was considerably more substantial.

Discussion

The inhibitory action of cranberry juice on bacterial adhesion, established in earlier studies,[22,23] was attributed to a high-molecular-weight component, termed NDM.[24] Our study of

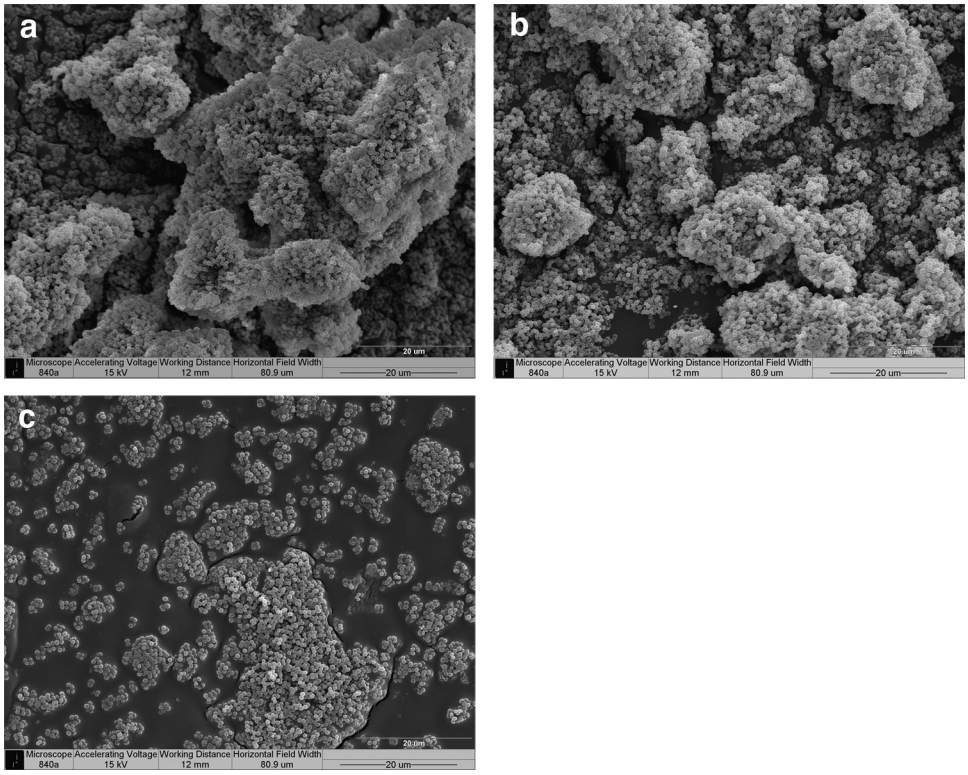
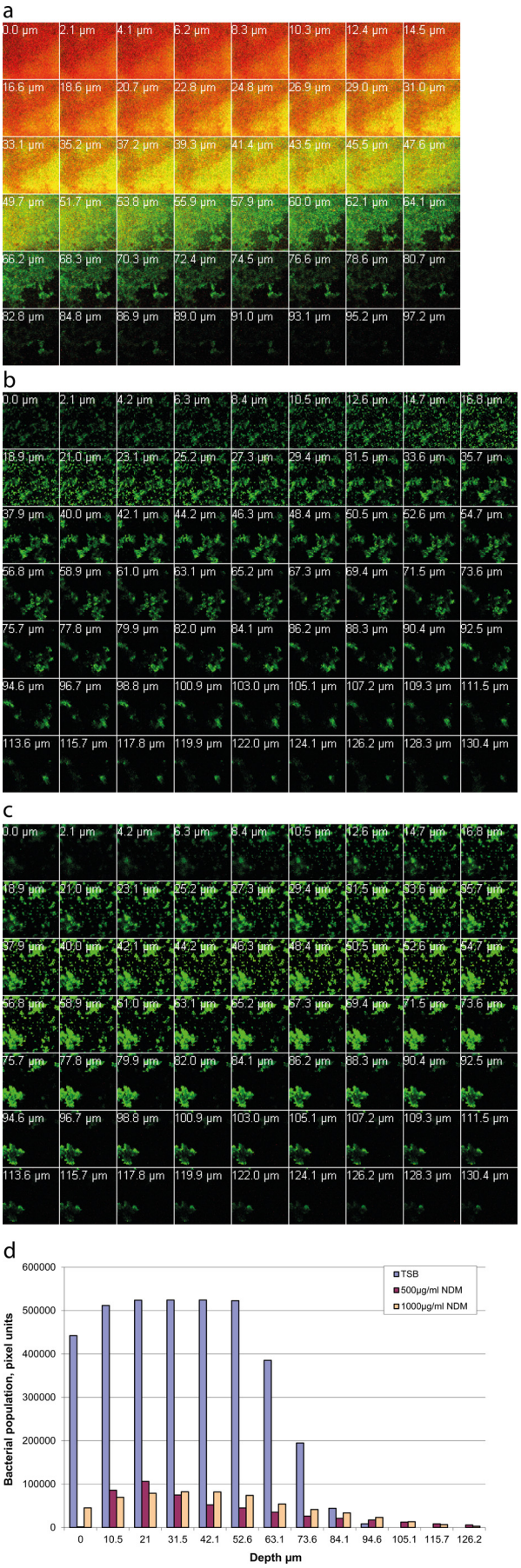


Figure 2. Representative SEM images of staphylococcal biofilm on contact lenses. The difference between the control (**a**) and treated samples is evident in the thickness of the biofilm. Notice the multilayered mushroom-shaped biofilm, clearly seen in the control. The lens surface is completely covered with bacteria. At 500 μg NDM per 1 mL TSB (**b**) biofilm is thinner, fewer layers can be seen, and lower mushroom-shaped protuberances are evident, and at several places the lens surface is exposed. At 1000 μg NDM per 1 mL TSB (**c**) the lens surface is clearly seen between sporadic microcolonies. No mushroom-shaped structures appear. All pictures are ×1500 magnification.



contact lens biofilm formation shows that NDM does not affect the viability of bacteria that form the biofilm but rather their ability to adhere to the soft contact lens material. The findings are consistent with previous studies showing that oligomeric proanthocyanidins derived from cranberry juice inhibited the adhesion of P-fimbriated *Escherichia coli*.[25]

Although the precise nature of NDM is not known, there is no doubt that it is a molecular species distinct from proanthocyanidins, as reflected by differences in solubility in various solvents, molecular weight, and mass spectroscopy.[17] Examining the toxicity of high-molecular-weight NDM is outside the scope of this manuscript, but it is worthwhile to point out that it was well tolerated in clinical trials where volunteers gargled NDM-containing mouth wash.[26] Furthermore, it is not clear how reports on PAC from other fruits such as grapeseed relate to cranberry PAC or NDM since NDM is different from cranberry PAC in a number of properties, the most important one is its molecular weight being a several orders higher than cranberry PAC.[17]

Cranberry juice cocktail contains approximately 1500 μg/mL NDM.[18] We found that the NDM concentration that inhibited biofilm formation was 500-1000 μg/mL (Fig. 1). NDM was also effective at concentrations above those found in cranberry juice, such as 2000 μg/mL. Regarding the issue of clumping, at the concentrations used we did not notice any clumping (data not shown), although at much higher concentrations (e.g., >3000 μg/mL) we did notice some clumping (data not shown).

NDM prevention of further expansion of biofilms in a dose-dependent manner suggests that NDM might potentially be used in contact lenses storage cases to prevent formation of biofilm.

Incidence of *S. epidermidis* keratitis may vary in studies of contact lens–associated infections.[27–29] However, it is the most prevalent species in in-dwelling medical device infections.[30] The strain used, RP62A, is a former clinical isolate from catheter-related infections and forms very good biofilms; however, further studies using different clinical isolates of staphylococci are needed to confirm these conclusions. The rationale of using TSB as a growth media is to provide optimal growth conditions. It is assumed that under nonoptimal conditions bacteria are expected to grow slowly, and therefore the effect of NDM might be undermined or require longer periods (e.g., days) for demonstrating an effect.

Our electron microscope evaluation of the contact lenses treated with NDM confirmed the reduction in bacterial adherence (Fig. 2). By further applying the vital dye, with confocal microscopy, we could evaluate the viability of bacteria inside the thick biofilm. We found that the majority of bacteria in the biofilm formed without NDM were dead (stained red by pro-

FIGURE 3. Staphylococcal biofilm on contact lenses, visualized by fluorescence vital dye. Images were acquired by Carl Zeiss LSM with a 20× objective lens. The images represent a z series, at 2.1 μm intervals. (**a**) Control, biofilm not treated with NDM, forms a thick layer of bacteria, covering the lens surface in a 9-μm–deep biofilm, spread almost evenly on the lens. The lower layers of biofilm bacteria are dyed red and therefore dead. (**b**) 500 μg/mL NDM-treated biofilm. The height is greater than that of the control, 130 μm deep, and dead bacteria are scarcely seen, even in the deeper layers. Also the biofilm is not as regular as in the control, and it comprises mostly mushroom-shaped protrusions, which are taller than the control. (**c**) 1000 μg/mL NDM-treated biofilm. The height is the same as in 500 μg/mL NDM, standing at 130 μm, and dead bacteria are scarcely seen, even in the deeper layers. As in 500 μg/mL NDM, the biofilm is not as regular as in the control, and it comprises mostly mushroom-shaped protrusions. At this concentration of NDM it was harder to find fields in which these protrusions are evident.

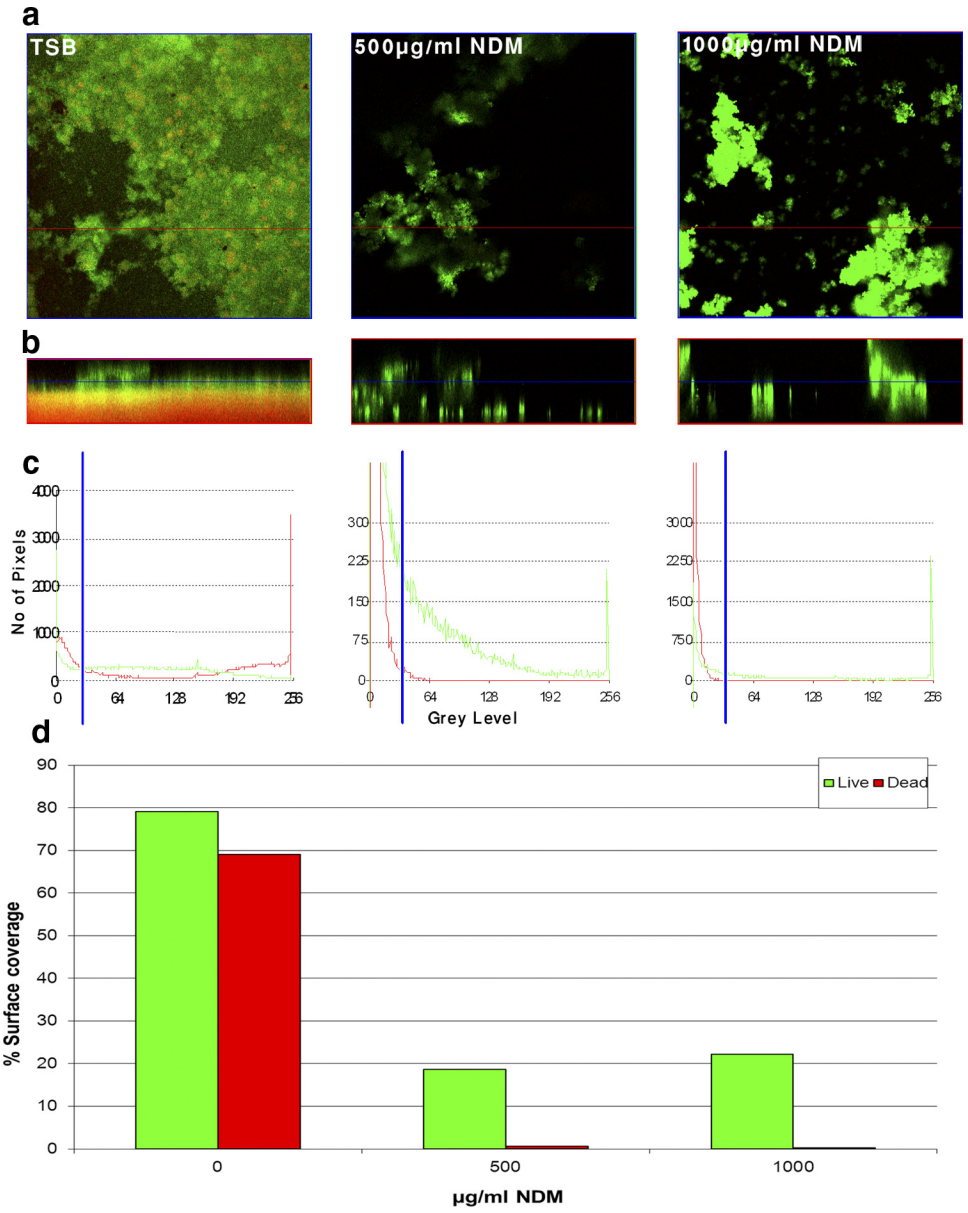


FIGURE 4. Area analysis of biofilm. The images were acquired at by using identical LSM settings with a ×20 objective lens. The larger images (**a**) represent a compressed *z* series, where multiple *x-y* planes from top to bottom of the biofilm are combined. The smaller images (**b**) represent *x-z* (side) views of the biofilm at the location indicated by the *red line*. (**c**) Histograms of the same *x-z* view, giving a visual representation of the red-to-green ratio in the depth of each sample. (**d**) The percentage of the pixel coverage of live (*green*) or dead (*red*) bacteria, after 30 gray-level threshold reduction (*blue line*; calculated with Matrox Inspector).

pidium iodide), a finding consistent with other studies,[31] whereas almost all the bacteria in the rather thinner biofilm formed in the presence of NDM were alive. Thus, the bacteria that did not detach from the biofilm formed on the contact lens surface were probably reacting to growth conditions on the contact lenses. The differences between Figures 2a, 2b, and 2c are not reflected in Figure 1. The method of preparing the samples for each of the experiments was very different. Loosely bound bacteria in Figure 1 remained associated with the biofilm and counted, whereas in electron microscopy, the harsh fixation treatment needed has removed any biofilm that was not firmly attached, such as biofilm treated with NDM. These differences in method would account for the differences observed. It is also possible that this effect may result from the insensitivity of using a scraping or turbidometric method for assaying biofilm. In confocal microscopy, the dyeing preformed was gentle and allowed the weaker, loosely attached, NDM-treated biofilm to be photographed as a whole.

The growth potential of any bacterial biofilm is limited by the availability of nutrients in the immediate environment, the perfusion of those nutrients to the cells within the biofilm, and the removal of waste products.[31] Other factors that control biofilm maturation include internal pH, oxygen perfusion, carbon source, and osmolarity.[32,33] Thus, the NDM-induced disturbance of attachment and growth of bacteria cause the biofilm to be thinner and consequently more permeable to intake of nutrients and disposal of waste. This would result in a lower percentage of dead bacteria.

When dealing with infected biomedical devices, including extended wear soft contact lenses, it is important to recognize that bacteria present in a mature biofilm behave quite differently from their planktonic counterparts. In particular, biofilm organisms are far more resistant to antimicrobial agents than organisms in suspension.[31] In some extreme cases, the concentrations of antibiotics required to achieve bactericidal activity against adherent bacteria of a biofilm can be three to four orders of magnitude higher than that needed for planktonic bacteria, depending on the species-drug combination.[34,35]

Further studies are necessary to test the anti-adhesion effect of NDM on biofilm formation on different contact lens polymers and different storage cases materials, as well as its addi-

tive bactericidal effect in combination with different antibiotic formulations.

In summary, the present study revealed that NDM reduces the formation of biofilm on soft contact lenses. This has important implications for the prevention of contact lens–related corneal infections caused by different bacteria, including *S. epidermidis*, which may adhere to soft contact lenses as well as their storage cases.[36–38]

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