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Role of Silver Ions in Destabilization of Intermolecular Adhesion Forces Measured by Atomic Force Microscopy in *Staphylococcus epidermidis* Biofilms

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In this paper, we report on the potential use of atomic force microscopy (AFM) as a tool to measure the intermolecular forces in biofilm structures and to study the effect of silver ions on sessile *Staphylococcus epidermidis* cell viability and stability. We propose a strategy of destabilizing the biofilm matrix by reducing the intermolecular forces within the extracellular polymeric substances (EPSs) using a low concentration (50 ppb) of silver ions. Our AFM studies on the intermolecular forces within the EPSs of *S. epidermidis* RP62A and *S. epidermidis* 1457 biofilms suggest that the silver ions can destabilize the biofilm matrix by binding to electron donor groups of the biological molecules. This leads to reductions in the number of binding sites for hydrogen bonds and electrostatic and hydrophobic interactions and, hence, the destabilization of the biofilm structure.

Microorganisms attach to living and nonliving surfaces, including those of indwelling medical devices and potable water system piping, and form biofilms that consist of extracellular polymers. Biofilms are the source of free-floating bacteria in drinking water, which poses a health risk (5). In biomedical areas, biofilms are a common source of medical device-related infections, such as acute bacterial infection of dental plaque (33), catheter-related bloodstream infection (7, 9, 34), and heart valves endocarditis (22). Although antibiotics like vancomycin and tobramycin (8, 23) are used to treat these infections, most of these infections persist. The use of antimicrobial agents and antibiotics for the treatment of these infections is limited, as the biofilms create an environment that enhances antimicrobial resistance (3, 16, 19). In general, the most important contribution of biofilms' antimicrobial resistance can be related to the properties of extracellular polymeric substances (EPSs), such as diffusion, sorption, water binding, mass transport, and mechanical stability. The EPSs of biofilms contain considerable amounts of polysaccharides, proteins, nucleic acids, and lipid (30), which are responsible for maintaining the structural integrity of the biofilm and providing an ideal matrix for bacterial cell growth (15). Intermolecular interactions between the various functional groups within these macromolecules serve to strengthen the overall mechanical stability of the EPSs and, hence, the survivability of the enclosed microorganisms.

It is reported that when silver ions bind to biological molecules containing thio, amino, carboxylate, imidazole, or phosphate groups, they inhibit activities that are vital to the bacteria's regulatory processes and cause bacterial inactivation (26, 27). Silver ions also act by displacing other essential metal ions, such as Ca^{2+} and Zn^{2+}. Silver cations exhibit broad antimicrobial action at low concentrations, and they are already being used for the treatment of burn wounds (24) and traumatic injuries (6). Silver ions also display low levels of toxicity to humans (1) and are safe agents for the removal of biofilms. Based on this reactivity of silver ions with the electron donor groups, we believe that silver ions have a considerable effect on the overall stability of EPSs, and the forces involved in EPS formation need to be investigated. As the EPSs play such an important role in the survivability of biofilms, the purpose of this study is to focus on the strategy of breaking up the EPS by the reactive action of silver ions and to investigate the role of silver ions in destabilizing the intercellular adhesion forces within *Staphylococcus epidermidis* biofilms by using atomic force microscopy (AFM).

MATERIALS AND METHODS

Bacterial strains. In this study, two different strains of *S. epidermidis* were used: RP62A (CIP 105777; purchased from the Institut Pasteur, France) and *S. epidermidis* 1457 (supplied by the Nebraska Public Health Laboratory, University of Nebraska Medical Center, Omaha). RP62A was characterized by accumulation-associated protein, which plays an important role in the accumulation of multilayer clusters during biofilm formation (11, 28). Strain 1457 produces the polysaccharide intercellular adhesin during the accumulation phase of biofilm formation (17, 18).

Cultivation of *S. epidermidis* biofilm. The strains were frozen in 20% glycerol solution and were stored in liquid nitrogen. When they were required, the frozen strains were thawed at 37°C before they were transferred into 10 ml of Trypticase soy broth (TSB; Difco) for incubation overnight. The culture was harvested during mid-exponential phase and washed three times in phosphate-buffered saline (PBS; pH 7.4). The pellets of the strains were prepared by centrifugation at 10,000 × *g* and suspended again in PBS. Freshly prepared bacteria were added

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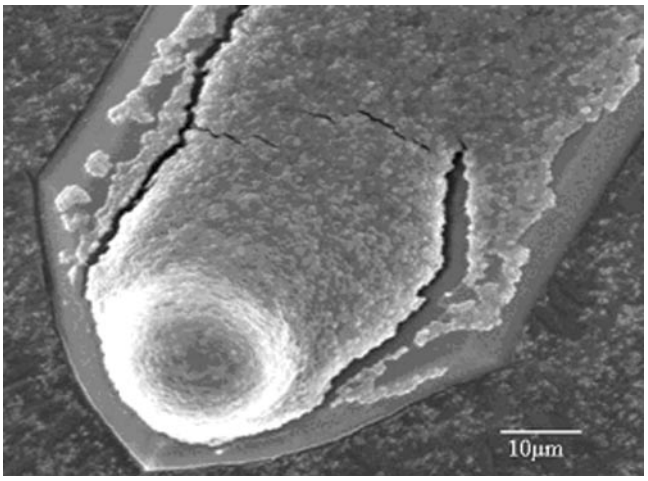

FIG. 1. SEM image of AFM cantilever tip coated with *S. epidermidis* biofilm.

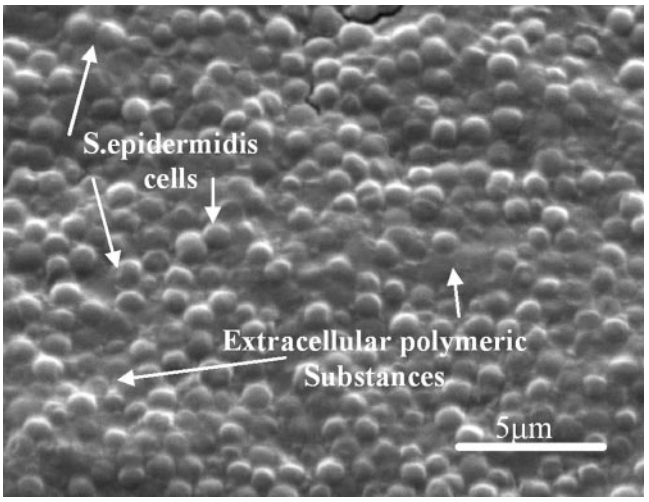

FIG. 2. Silicon surface coated with *S. epidermidis* biofilm.

to TSB diluted 1:50 and were incubated in a 48-well culture plate at 37°C. Polycarbonate slides (1 cm by 1 cm) were also added to the wells. A constant shaking motion was introduced to the culture system to induce initial adhesion and subsequent biofilm proliferation. The *S. epidermidis* biofilm was allowed to grow on the polycarbonate slides as well as the base of the wells for at least 2 days before experiments were performed.

***S. epidermidis* cell viability study.** The sessile cell viability of *S. epidermidis* RP62A biofilms at different times of growth (4, 7, and 12 days) was examined after the addition of silver ions for 5, 10, 30, and 60 min. The supernatant was first removed, and the attached biofilm was gently rinsed three times with autoclaved PBS. The PBS (0.1 M; pH 7.4) was prepared with 3.116 g/liter of Na_2HPO_4 (Sigma) and 22.115 g/liter of NaH_2PO_4 (Sigma). Silver ions (50 ppb; J. T. Baker) were then added to the wells. After different contact times, the culture plate was sonicated for 15 min before serial dilution of the control and the silver-treated sessile cells was carried out. The cells were plated on Trypticase soy agar in triplicate, and they were incubated at 37°C for 2 days before they were counted.

Scanning electron microscopy (SEM) of *S. epidermidis* biofilms. After growth for 4, 7, and 12 days, the polycarbonate slides were removed and gently rinsed three times in autoclaved PBS to remove any remaining nonadherent bacteria. The slides were then incubated again at 37°C in PBS with and without the addition of 50 ppb of silver ions for a contact time of 60 min. The slides were again removed and rinsed three times in autoclaved PBS. The slides were then gently air dried and stored at 4°C. The biofilms' structures were subsequently observed with a JSM-5600 electron microscope (JEOL, Japan). All images were taken at an operating voltage of 10 kV, with secondary electron imaging used for better visualization of the surface morphology.

Preparation of *S. epidermidis*. Coated tip and substrate silicon wafers of 1 cm by 1 cm were used as substrates to coat the layer of *S. epidermidis*. The wafers were first cut and cleaned with concentrated sulfuric acid and 30% hydrogen peroxide (7:3; volume ratio) at 80°C for 45 min. After that, a drop of polyethyleneimine (PEI) solution was allowed to adsorb on the substrate for 3 h before the solution was decanted and the substrate was rinsed in deionized water (4). After this process, the PEI-coated substrates were air dried. Subsequently, *S. epidermidis* cells were manually transferred onto the PEI-coated AFM tip by using a 10-μl pipette and observation under an optical microscope. The *S. epidermidis*-coated tip was then viewed with a high-resolution microscope (model INM100; Leica) to ensure complete coverage of the bacterial cells over the tip, and the coated tips were then stored at 4°C. The SEM images of the coated AFM tip and substrate are shown in Fig. 1 and Fig. 2, respectively. A clear distinction between the *S. epidermidis* cells and the EPSs can be seen in these figures.

Atomic force microscopy. The intermolecular force measurements were carried out with a Multimode Nanoscope IV AFM (Digital Instruments). All force measurements were performed in PBS liquid medium with an AFM fluid cell between the *S. epidermidis*-coated AFM tips and the substrate. The AFM experiments were carried out by engaging the AFM cantilever tip without contacting the substrate, and then the tip was moved toward the substrate in 50-nm increments. For the control sample, the AFM force measurements of 100 readings in PBS solution were taken for each *S. epidermidis*-coated cantilever tip and the substrate. After that, silver ions (50 ppb) were added to the same sample to get the force-distance (F-D) curve in the presence of silver ions.

The silver ions were diffused into the core of the *S. epidermidis*-coated AFM tip and the substrate within the given contact time of 2 min. The cantilever-substrate combination was not changed during the experiments in order to eliminate the possibility of varying the spring constant among the AFM cantilevers. A total of four sets of force-distance measurements were obtained from the AFM tips and the surfaces coated with *S. epidermidis* RP62A (characterized by its accumulation-associated protein) and *S. epidermidis* 1457 (characterized by its polysaccharide intercellular adhesin). Initially, the tip deflection data were acquired in nanometers, and these were later converted to force (nN) by using image-processing software, SPIP (Image Metrology, Denmark).

RESULTS

Effects of silver ions on sessile *S. epidermidis* cell viability and *S. epidermidis* biofilm structure. Figure 3 shows the SEM images of the biofilm structure taken at different days of growth with and without the addition of silver ions. At day 4, the control sample had well-structured growth of approximately 10 to 25 μm in width. After the addition of silver ions and within 60 min of contact, the overall structure of the biofilm became partially destroyed and the inner structure of biofilm was exposed. There were also significant amounts of EPSs around the damaged biofilm colony, confirming the effect of silver ions on the grown biofilm matrix. At 7 and 12 days of growth, the biofilms were mature, had established themselves structurally, and had grown to 30 to 40 μm and 60 to 100 μm in width, respectively. Although the biofilms were structurally held with a thick layer of extracellular matrix, significant changes in their overall structures were still observed after treatment with silver ions. The silver ion-treated biofilm matrix was generally observed to lose its compactness, leading to the exposure of its inner core. As shown in Fig. 4, the viabilities of the bacterial cells were not affected by the low 50-ppb concentration of silver ions for 60 min of contact. Removal of more that 1 log CFU/cm^2 was not attainable even after the contact time of the silver ions with the biofilm matrix was increased to 60 min, but there was a disruptive change to the biofilm structures after treatment with silver ions.

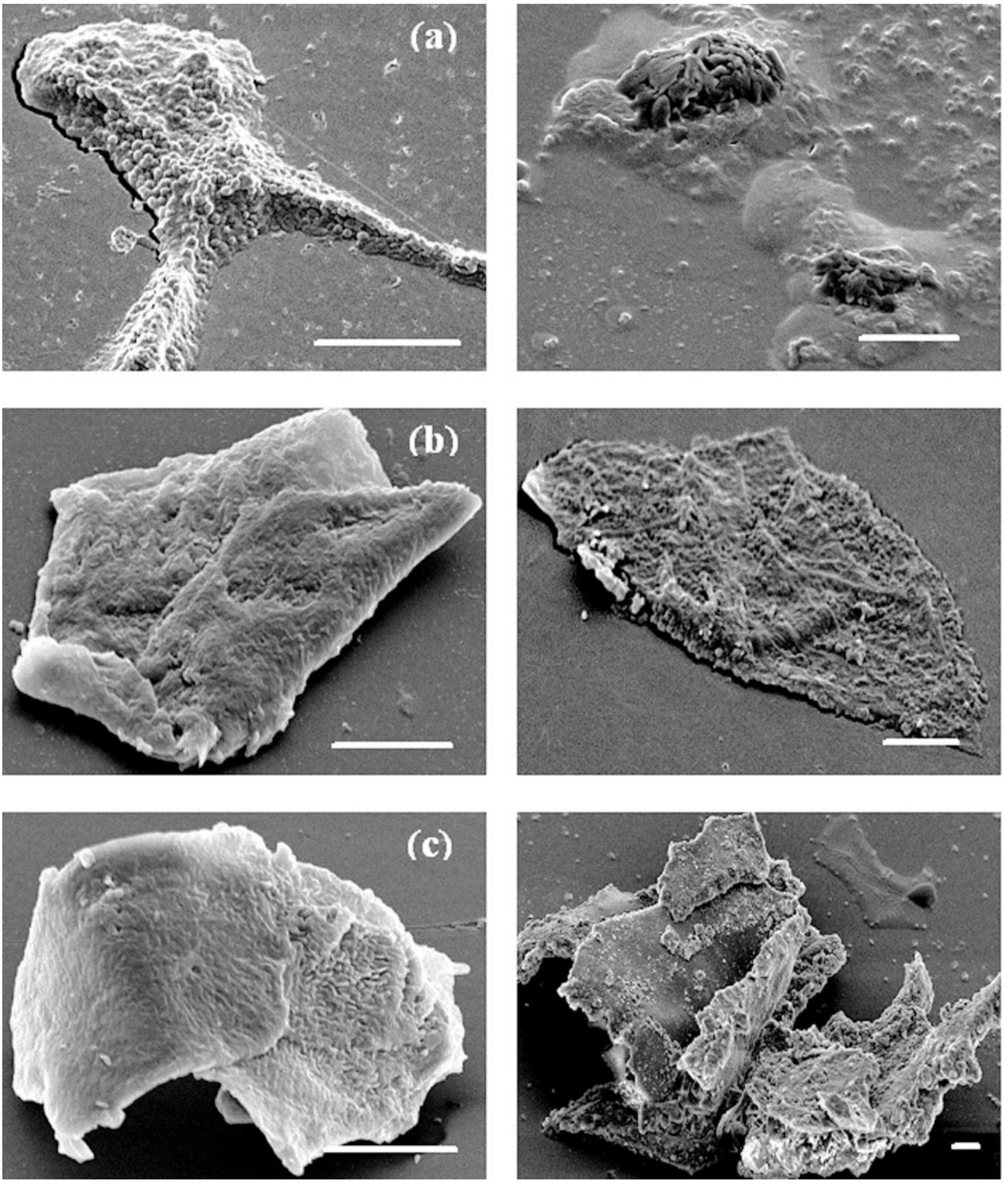

FIG. 3. SEM images of biofilm structures of the control samples (left panels) and the silver ion-treated samples (right panels) at the 4th (a), 7th (b), and 12th (c) days of growth. Disruptive changes in the biofilm structure can be observed after reaction with the silver ions. Bars, 10 μm.

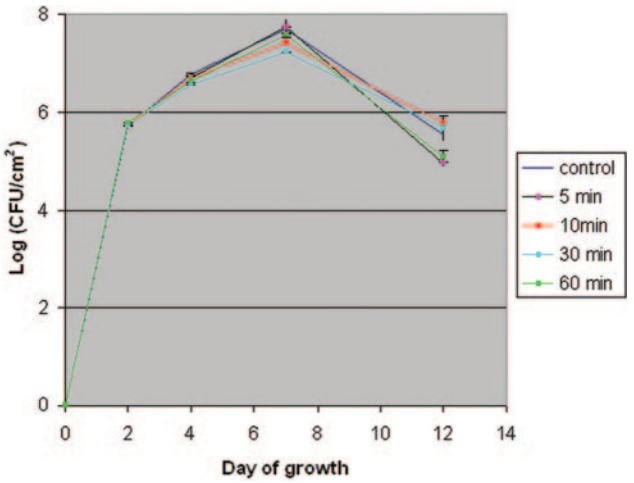

FIG. 4. *S. epidermidis* sessile cell viability before and after the addition of 50 ppb of silver ions with different contact times. There are insignificant effects from the action of the silver ions at different contact times and different times of biofilm growth.

AFM study of intermolecular adhesion forces within biofilm matrix. Figure 5 shows the typical F-D curves for the control sample in a normal PBS environment, and Fig. 6 shows the F-D curve under the influence of 50 ppb of silver ions. These F-D curves are shown as tip-approaching curves and tip-retracting curves. The graphical illustrations in Fig. 5 and Fig. 6 explain the variation and the magnitude of forces experienced by the AFM cantilever tips as they approached the biofilm-coated substrate, became embedded in the biofilm matrix, were gradually pulled away from the biofilm, and finally released from the biofilm surface.

In general, the F-D curves for both the control sample and samples to which silver was added displayed similar responses during the approach stage of the cantilever tip toward the biofilm-coated substrate. The tip initially undergoes negligible forces (labeled A in Fig. 5 and Fig. 6) until it becomes close to the substrate. When the tip nears the surface (labeled B in Fig. 5 and Fig. 6), it is then repelled by a positive net force due to the combination of electrostatic double-layer effect and the steric effect by the substrate. Immediately after that, the tip undergoes a strong attraction as a result of a negative net force toward the substrate, giving a sharp slope, as shown in Fig. 5. Further engagement of the tip into the substrate causes the tip to touch the biofilm matrix physically (labeled C). It is of note that in the compliance region, where the tip is driven into the coated surface, the curve's gradient is not constant but increases nonlinearly; this is a characteristic of soft surfaces. Therefore, we confirmed that the force measurement is obtained from the soft surface of the EPSs and not the underlying hard silicon surface.

However, the retracting portion of each curve is different; the tip-retracting part of the curve (labeled E in Fig. 5) stretches over a long range of about 30 nm and has a single breakage point recording (adhesion force) of 16 nN due to the release of the tip from the coated substrate. The adhesion force represents the magnitude of a combination of intermolecular forces within the EPS between the coated tip and the substrate. After the addition of 50 ppb of silver into the fluid environment, the tip-retracting part of the curve occurs at multiple locations (between points E and H in Fig. 6). The forces measured at these locations are found to be between 0.1 nN and 4.0 nN. These forces are much lower than the adhesion force of the control sample and stretch over a much shorter distance of 5 to 15 nm. This means that in the control sample, the tip is retracted for 30 nm before the EPS is disturbed, whereas in the silver ion-treated sample, the tip is retracted only 5 to 15 nm.

Table 1 shows the quantitative percent distributions of the intermolecular adhesion forces of the *S. epidermidis* RP62A and 1457 biofilms obtained from 100 similar F-D curves. Generally, a wide range of intermolecular adhesion forces is recorded within the heterogeneous EPSs. These force distributions within the EPSs do not follow a normal distribution but are skewed slightly, indicating their uneven distributions. Under normal PBS conditions, intermolecular adhesion forces in the ranges of 0.3 nN to 19.4 nN and 1.0 nN to 21.2 nN are recorded for *S. epidermidis* RP62A and *S. epidermidis* 1457, respectively. After the addition of silver ions, their intermolecular adhesion forces dropped to ranges of 0.2 nN to 6.7 nN and 0.1 nN to 13.9 nN for *S. epidermidis* RP62A and *S. epidermidis* 1457, respectively. This is accompanied by a percent distribution shift of the intermolecular adhesion forces toward the lower force range (0.1 to 4nN). In the range of 0 to 2 nN, the increases in the force distributions are 26% and 36% for *S. epidermidis* RP62A and *S. epidermidis* 1457, respectively. Also, there is a decrease in the distribution of intermolecular adhesion forces in the higher force range of 4 nN and above for both the *S. epidermidis* RP62A and the *S. epidermidis* 1457 biofilm structures.

DISCUSSION

Antimicrobial ineffectiveness of silver ions towards sessile cells at 50 ppb. While silver ions at ppb concentrations are effective antimicrobial agents against most planktonic cells (12, 25), they are have been shown to be ineffective against cells residing within the biofilm, as shown in Fig. 4. Even after the contact time is increased, there are still no significant changes in their viabilities. One possible reason is that the silver ions are trapped inside the biofilm matrix. As such, the silver ions are active only at the periphery of the biofilm and therefore are ineffective at penetrating the deeper core of the biofilm matrix, where the bulk of the bacterial cells are usually present (3, 19). Another explanation is the complexation of silver ions after their interactions with molecules in the EPSs (16, 19), which leads to inactivation of their antimicrobial activities, as they exhibit antimicrobial activity only when they are in the ionic form (29). This inefficacy of silver ions at a low concentration against sessile cells is also consistent with the findings of a previous study (20). This suggests that a small dosage of silver ions is insufficient to release excess unbound silver ions for antimicrobial action. This also implies that low concentrations of silver ions are unsuitable for the treatment of biofilm infections. Although higher silver concentrations have increased effectiveness against sessile cells (2, 20), they nevertheless face the challenge of maintaining their ionic form in applications containing large amount of halides and other ions, like Cl^-,

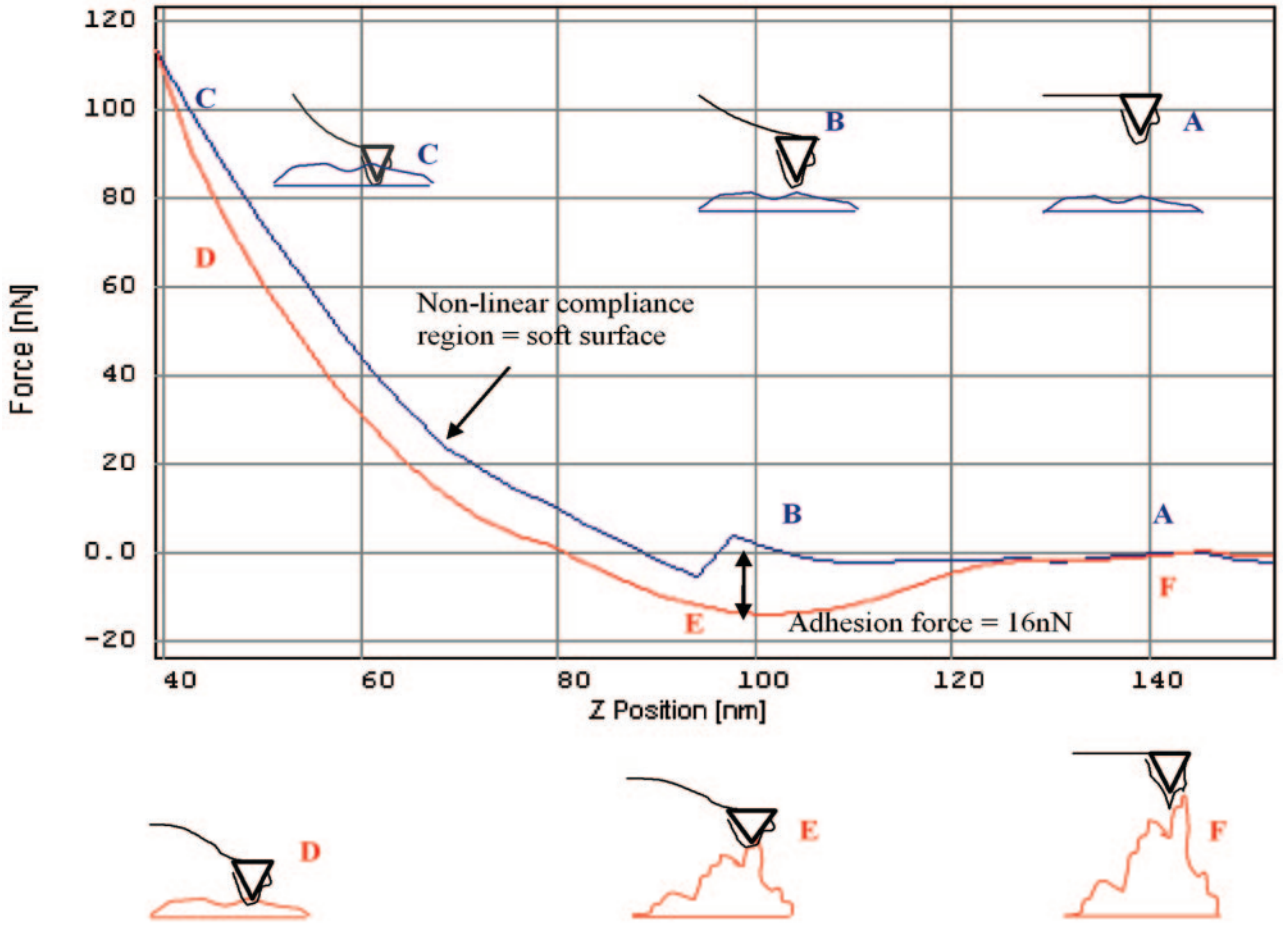

FIG. 5. AFM force-distance curve illustrating the single breakage point of the interaction between the EPSs in a normal PBS environment. The blue line accompanied by labels and illustrations A to C represents the tip-approaching curve, whereas the red line accompanied by labels and illustrations D to F represents the tip-retracting curve. The labels and illustrations indicate the following: at point A, the tip experiences negligible force; at point B, the tip experiences repulsion, followed by a strong attraction force by the coated substrate; at point C, the tip gets embedded into the EPS; at point D, the tip begins retracting from the biofilm-coated substrate and the cantilever bends differently; at point E, the tip is being pulled away from the coated substrate over a distance of 30 nm; and at point F, the tip is fully released from the coated substrate and has gone back to its initial approach position.

HCO_3^-, and CO_3^-, and protein anions (13, 27) due to the production of the insoluble silver salt, which causes silver ion inactivity.

Disruption of intermolecular adhesion forces within *S. epidermidis* biofilm matrix by using SEM and AFM study. Although the low silver ion concentration has ineffective antimicrobial activity for the treatment of biofilms, it destabilizes the EPSs within the biofilm matrix. The SEM images of the silver-treated sample taken on different days indicate that the biofilm matrix is less compact, with its inner core exposed (Fig. 2). We believe that the entrapment and complexation of silver ions with EPSs can play an important role in the destabilization of the biofilm matrix. Generally, EPSs contain a wide variety of macromolecules and ions (30), which have multiple forms of interactions, like hydrogen bonding between electronegative atoms and polymer bridging between divalent cations. The combination of these interactions contributes to the biofilm's overall cohesiveness. As silver ions are highly reactive and bind strongly to the electronegative electron donor groups containing oxygen or nitrogen (26) in the EPSs, we suggest that they must be able to bind to biological molecules like proteins and polysaccharides within the EPSs. Therefore, the addition of silver ions into the EPSs causes the number of possible intermolecular interactions between these molecules to decrease. The net effect is two separate arms of the interacting polymer chains dangling within the biofilm matrix. We therefore suggest that the combination of these mechanisms may ultimately lead to the breakdown of the EPS structure and, hence, the weakening of the biofilm matrix.

From Fig. 5 and 6 we have observed that there is only one distinct breakage point (point E in Fig. 5) under normal PBS conditions, whereas there is a series of multiple breakage points (from points E to H in Fig. 6) for the silver-treated sample. Each breakage point represents the intermolecular adhesion force as well as the interacting distance between the EPSs and provides an excellent indication of the interaction strength between the EPSs. Under normal physiological conditions, the maximum intermolecular adhesion forces for *S. epidermidis*

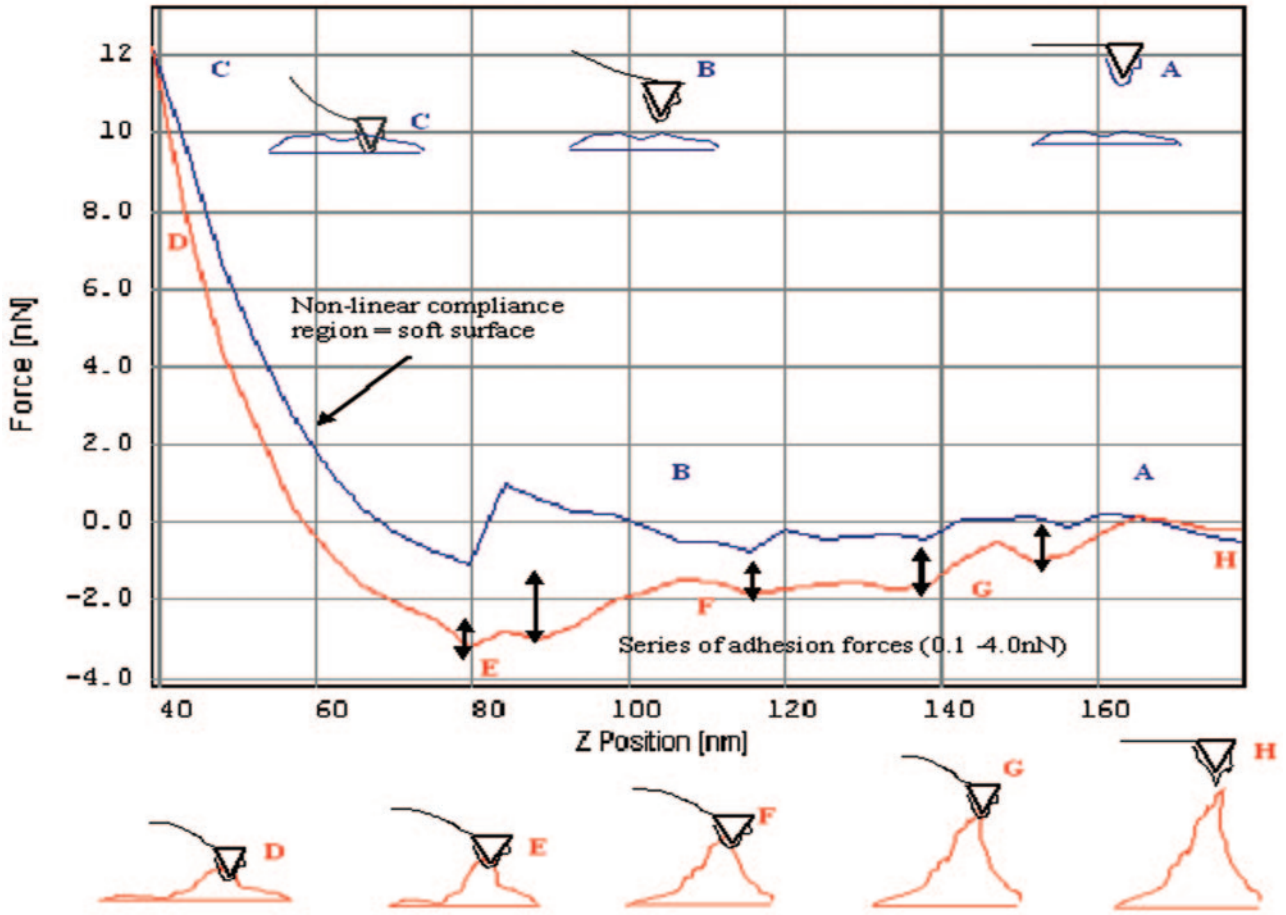

FIG. 6. AFM force-distance curve illustrating the multiple breakage points of the interaction between the EPSs after the addition of 50 ppb of silver ions. The blue line from points A to C represents the tip-approaching curve, whereas the red line from points D to H represents the tip-retracting curve. The labels and illustrations indicate the following: at point A, the tip experiences negligible force; at point B, the tip experiences repulsion, followed by a strong attraction force by the biofilm-coated substrate; at point C, the tip gets embedded into the EPSs; at point D, the tip begins retracting from the substrate and the cantilever bends differently; from points E to G, the tip undergoes multiple breakage, with the EPSs recording a weak interaction force and a smaller interacting distance; and at point H, the tip is fully released from the substrate and has gone back to its initial approach position.

RP62A and *S. epidermidis* 1457 are 19.4 nN and 21.2 nN, respectively but they decrease to 6.7 nN and 13.9 nN, respectively, after the addition of 50 ppb of silver ions. Therefore, this finding suggests that the silver ions destabilized the grown biofilm matrix. The shorter interacting distance of 5 to 15 nm in the silver-treated sample compared to an interacting distance of 30 nm in the control sample further suggests that the EPSs are no longer as cohesive and can be broken easily. We have also observed that the addition of the silver ion causes a shift in the distribution of the intermolecular adhesion force (Table 1) within the EPSs toward the lower range (0.1 nN to 2 nN). This increase in the distribution of forces at the lower range is also accompanied by a corresponding decrease in the distribution of forces in the higher force range. These observations show that the silver ion indeed has a destabilizing effect on the intermolecular adhesion forces, resulting in a cracked biofilm matrix, which is being held together by a number of weak interaction forces. Hence, we propose that the destabilizing effectiveness of the silver ions on both *S. epidermidis* RP62A and 1457 biofilms may be useful for tackling *S. epidermidis* biofilm-associated problems, like infections of implanted medical devices (21). Apart from the present applications, such as coating of a catheter with silver ions to avoid bloodstream infections (7, 32), a strategy of using the silver ions' destabilizing capability in combination with other drugs (10, 14) can be explored for the testing of antimicrobial effectiveness. This synergistic approach may work well, with the silver ions destabilizing the biofilm matrix to increase the biofilm's contact area and the antimicrobial agents subsequently killing the bacteria. Due to the risks of emerging multidrug-resistant pathogens, natural antimicrobial agents like usnic acid (10) and lactoferrin (14) may be potential choices for this purpose.

Conclusion. In conclusion, the potential use of AFM as a tool for the quantitative measurement of the intermolecular forces involved in biofilm formation and biofilm removal is demonstrated in this paper. For this purpose, we have coated the AFM tip and the silicon surface with an *S. epidermidis* biofilm to measure the intermolecular forces and to study the effects of silver ions on the biofilm using AFM. We have observed that in the presence of silver ions, there is a shift in the distribution of the intermolecular adhesion force within the

TABLE 1. Percent distributions of intermolecular adhesion forces of *S. epidermidis* RP62A and 1457 before and after treatment with silver ions

Range of intermolecular adhesion forces (nN)	% Distribution of intermolecular adhesion forces			
	S. epidermidis RP62A		*S. epidermidis* 1457	
	PBS[a]	PBS and 50 ppb of Ag^{+b}	PBS[c]	PBS and 50 ppb of Ag^{+d}
0–2	42.4	68.4	5.5	41.6
2–4	32.5	23.8	22.7	37.4
4–6	9.9	7.3	24.1	15.9
6–8	5.8	0.5	16.4	2.3
8–10	3.7	0	10.9	1.4
10–12	3.1	0	5.5	0.5
12–14	0	0	7.7	0.9
>14	2.6	0	7.3	0

[a] Recorded maximum value is 19.4 nN.
[b] Recorded maximum value is 21.2 nN.
[c] Recorded maximum value is 6.7 nN.
[d] Recorded maximum value is 13.9 nN.

EPSs toward the lower range (0.1 nN to 2 nN). This increase in the distribution of forces at the lower force range is accompanied by a decrease in the distribution of forces in the higher force range (4 nN and above); this proves the destabilizing effect of silver ions on the biofilm matrix. It should be noted that the low concentration of 50 ppb of silver ions is ineffective against sessile cells. We believe that the interaction of silver ions with macromolecules through their complete discretion and the displacement of divalent cations in the EPSs play a vital role in this destabilization process. Since macromolecules such as proteins and ions have a large proportion of dry biofilm mass (31), they make a tremendous contribution to the overall cohesiveness of biofilms. The study on the use of silver ions' ability to break up and disrupt biofilm structure is important for devising novel and efficient strategies to remove biofilms. Therefore, we suggest that to enhance biofilm removal, we need to use a synergistic approach to use the silver ions' destabilizing capability.

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