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Abstract: The antibacterial characteristics of triclosan- or bronopol-coated and plasma-modified polyethylene (PE) are investigated. The modified PE samples exhibit excellent bactericidal effects against *Escherichia coli* and *Staphylococcus aureus* even when the bacteria concentration in the suspension is 10^6 colony forming units (CFU)/mL. However, when the concentration exceeds 10^8 CFU/mL, the materials fail to develop noticeable resistance to large amount of bacteria because of the formation of a bacterial biofilm on their surfaces. The PE treated by this relatively simple technique possesses excellent antimicrobial properties and is useful in biomedical and disinfection applications because the bacteria concentrations in most situations are well below 10^6 CFU/mL. © 2006 Wiley Periodicals, Inc. *Biopolymers* 83: 62–68, 2006

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INTRODUCTION

The importance of bacterial adherence and biofilms in nosocomial infection has spurred research activities on antimicrobial agents.^{1–5} Polymers that are one of the most important biomaterials are commonly surface modified for sterilization purposes in many environmental and medical applications.⁶ Conventional surface modification techniques are usually based on the incorporation of a leachable antiseptic into a polymeric surface coating.^{7,8} Unfortunately, these loosely bound antimicrobial agents easily leach into the environment and cause side effects to humans as well as the environment. A better approach is covalent surface functionalization using antimicrobials that do not leach into the environment. It has been found that covalent immobilization of antimicrobial bronopol or triclosan by means of plasma immersion ion implantation (PIII) can yield excellent microbicidal effects on the polymer surfaces against both Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*.^{9–11} This approach is relatively simple and produces little chemical pollution, good surface conformity, and uniformity. Cosmetically, PIII does not alter the appearance of polymers such as poly vinyl chloride and polyethylene.^{9–13}

In contact with different cell concentrations, these modified polymers usually exhibit different degrees of antimicrobial effects because biofilm-grown bacteria have properties that are different from those of planktonic bacteria. One such property is the increased resistance to antimicrobial agents.^{14–18} In the work reported here, we investigate the microbicidal properties of the plasma-treated polyethylene (PE) in solutions with variable cell concentrations and the evolution of the bacterial biofilm on the modified surface.

EXPERIMENTAL

Antimicrobial Sample Preparation

Medical-grade PE samples (LDPE, 51215B Beijing Huaer Co., Ltd.) were loaded into the plasma immersion ion implanter.^{10–13,19} An initial O₂ plasma treatment was performed under optimal conditions determined in previous experiments. The conditions were bias voltage = –12 kV, voltage pulse width = 20 μs, pulsing frequency = 30 Hz, gas flow = 20 standard cubic centimeter (sccm), radio frequency (RF) power = 1000 W, and treatment time = 30 min.^{9–11} Subsequently, the samples were taken out and immediately precoated with an antimicrobial agent, triclosan or bronopol (Tian Jing Well-Real Chemical Technology, Co., Ltd.). Finally, they underwent argon plasma immersion ion implantation to ensure that the antibacterial reagent bonded

well onto the PE surface.¹⁰ Different processes were used for different samples. Sample 1 was the untreated PE (control). Sample 2 was treated with oxygen plasma, coated with triclosan, and then treated with argon plasma. Sample 3 was processed similarly to sample 2 except that the agent used was bronopol instead of triclosan.

Antibacterial Assays

The antibacterial performance against *Staphylococcus aureus* ATCC6538 (*S. aureus*, Gram-positive) and *Escherichia coli* ATCC10536 (*E. coli*, Gram-negative) was determined by the plate counting method.^{9,10,19} Seventy percent ethanol was first used to sterilize the samples and then a 0.2 mL solution of bacteria [$1 \sim 5 \times 10^5$ (CFU)/mL] was added onto the modified surface and covered by a PE film (4 × 4 cm). At a relative humidity (RH) of higher than 90% and a temperature of $37 \pm 1^\circ\text{C}$, the bacteria on the samples were incubated for 24 h. Afterwards, they were thoroughly washed with 20 mL of 0.87% NaCl solution that contains Tween 80 with a pH of 7.0 ± 2 . To observe the active bacteria, 0.2 or 0.02 mL of the washing solution was added into the different dishes containing the nutrient agar. After 24 h of incubation under similar conditions, the active bacteria were counted and the antibacterial effect was quantitatively determined using the following relationship⁹:

$$R (\%) = [(B - C)/B] \times 100$$

where R is the antibacterial effect (%), B is the mean number of bacteria on the control samples [colony forming units (CFU)/sample], and C is the mean number of bacteria on the modified samples (CFU/sample).

Bacteria Assays on Sample Surfaces

Two solutions with different bacteria concentrations were used to investigate the bacteria behavior on the modified PE surfaces. The cell concentration in the lower concentration solution was $1 \sim 5 \times 10^6$ CFU/mL, and the high bacteria concentration solution was obtained from the Luria–Bertani (LB) solution containing the highest concentration of bacteria after incubation for 36 h. With the exception of the different cell concentrations, similar processes were adopted.^{9,10,19–22} The samples were sterilized by 70% ethanol and cut into 16 pieces of approximately 2.0 cm². They were placed in three different flasks containing the bacteria suspensions and kept at ambient conditions. Three samples were taken out sequentially from the bacteria suspensions and gently rinsed thrice with a 0.87% NaCl solution containing Tween 80 at a pH of 7.0 ± 2 to remove bacteria not adhering to the surface. Thereafter, the adhered bacteria were detached from the samples in 10 mL of NaCl solution ultrasonically. The solution with the bacteria was used to determine the viable counts.^{9,10}

Sample Preparation for Scanning Electron Microscopy Observation

The samples were dipped in the cell suspensions, taken out, and placed in a 3% glutaraldehyde solution with 0.1M cacodylate

Table I Calculated Contact Angles and Surface Energies for Plasma-Modified Samples

Samples	Contact Angle (°)		Dispersion (γ_s^d) nJ/cm ²	Polar (γ_s^p) nJ/cm ²	Surface Energy (γ_s) nJ/cm ²
	Distilled Water	Glycerin			
Sample 1	94.7	83.9	16.39	4.13	20.52
Sample 2	63.2	55.7	18.06	21.30	39.36
Sample 3	64.4	47.1	33.55	12.33	45.88

dylate buffer for several hours to fix the bacteria on the sample surfaces. Subsequently, they were washed with a 0.05M cacodylate buffer and then doubly distilled water. To dehydrate the bacteria, they were washed by gradually increasing concentrations of alcohol up to 100% for 15 min. Finally, they were placed in hexamethyldisilazane (HMDS) for drying before they were studied by scanning electron microscopy (SEM).

Hydrophilicity Determination

Distilled water or glycerin was used to measure the surface angles by the sessile drop method using a Ramé-Hart (USA) instrument at ambient humidity and temperature.^{11,23,24} Each data point represents the average of five measurements conducted on different parts of the specimen for statistical accountability.

RESULTS AND DISCUSSION

Surface Hydrophilicity

Bacterial adhesion is thought to be the first of the two steps for biofilm formation, and the second stage is accumulation of the bacterial cells in the biofilm mass.¹⁷ When the microorganisms come in contact with the material surface, they are usually attracted or repelled, depending on the integral effects of the different interactions. From an overall physicochemical point of view,^{15,17} microbial adhesion can be mediated by nonspecific interactions with long-range characteristics including Lifshitz–van der Waals forces, electrostatic forces, acid–base interactions, and Brownian motion forces.¹⁵ In biological systems, hydrophobic interactions are usually the strongest of all long-range noncovalent forces.^{15,25–28} Hence, before the investigation of bacterial adhesion and biofilm in our study, the surface hydrophilicity of the triclosan- or bronopol-modified polyethylene is first evaluated. The substrate surface physicochemical characteristics are presented in Table I.^{11,18,23} The water contact angles acquired from samples 2 and 3 change from 94.7° to 63.2° and 64.4°, respectively. Their surface energies (γ_s) decrease to 39.36 and 45.88 nJ/cm² as well. Our results show evidence that plasma modification improves the hydrophilicity of the PE surface, and hydro-

philic bacteria will more likely adhere onto the modified sample surface based on previous studies.^{17,18}

Bacterial Biofilm on Modified Samples

In order to investigate the bacterial biofilm formed on the surface, we have chosen *E. coli* and *S. aureus* as the Gram-negative and Gram-positive bacteria. Suspensions with high cell concentrations were prepared to form better biofilms. As shown in Figures 1 and 2, the concentration of *E. coli* and *S. aureus* in the suspensions are about 10⁸ CFU/mL after incubating for about 36 h. Figures 3 and 4 show the bacterial biofilm formation on the samples. Each data point is the mean of four values in the figures. The results show that samples 2 and 3 do not possess antimicrobial properties under a high concentration of bacteria of 10⁸ CFU/mL. However, according to the antibacterial properties determined previously,¹⁰ both of them should have better antimicrobial performances (Table II). Based on results reported earlier,^{18,33} the mechanisms of microbial resistance include (a) limited diffusion of the antimicrobial agents through the biofilm matrix, (b) interaction between the antimicrobial agents and the biofilm matrix, (c) enzyme mediated resistance, (d) level

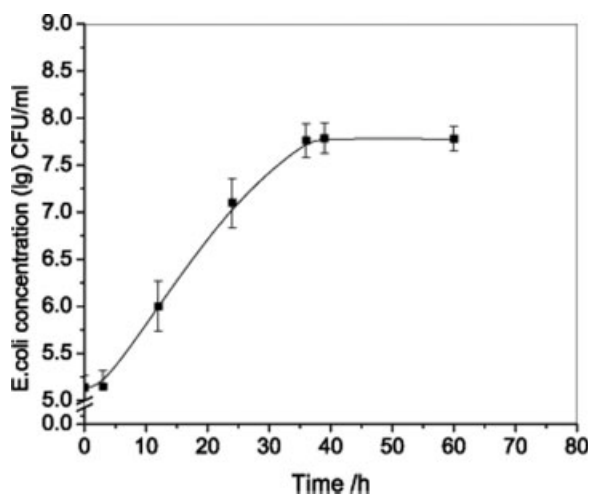


FIGURE 1 *Escherichia coli* growth curve in the LB solution.

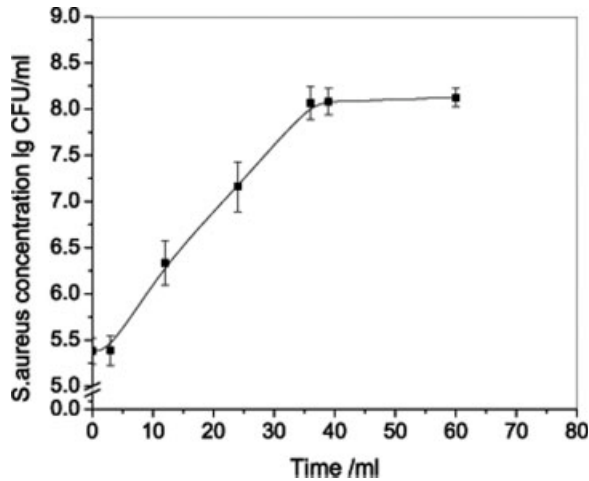


FIGURE 2 *Staphylococcus aureus* growth curve in the LB solution.

of metabolic activity within the biofilm, (e) genetic adaptation, and (f) efflux pump and outer membrane structure.

Because the antimicrobial agents are immobilized on the surfaces, the inferior antimicrobial properties observed in our experiments mainly stem from reduction of the interactions between the planktonic bacteria and antimicrobial agents. After the samples are placed in the cell suspension, a large amount of planktonic bacteria adhere on the modified surfaces in a short time, followed by reactions that kill most of the bacteria close to the surface. Therefore, the bacteria form a layer of biofilm on the surface giving physical protection between the planktonic bacteria and antimicrobial on the surfaces. Moreover, the antimicrobial activity of triclosan has been suggested to be concen-

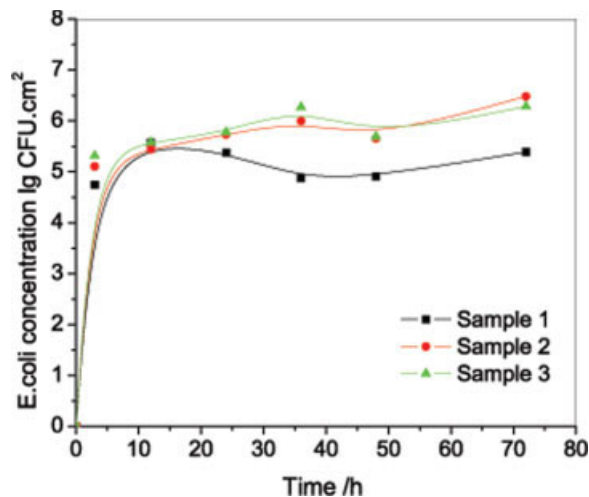


FIGURE 3 *Escherichia coli* biofilm growth curve on the samples in the cell suspension with about 10^8 CFU/mL.

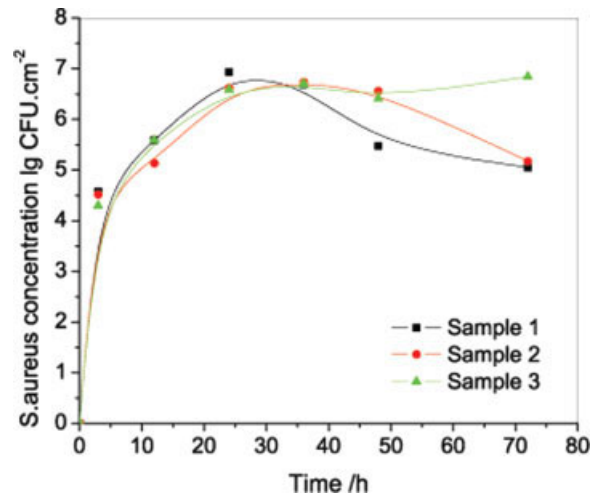


FIGURE 4 *Staphylococcus aureus* biofilm growth curve on the samples in the cell suspension with about 10^8 CFU/mL.

tration dependent and these antimicrobial agents are not capable of penetrating this barrier due to their immobilization. On the other hand, based on results reported previously,^{9,10,29-31} triclosan or bronopol acts as a nonspecific biocide by affecting the membrane structure and function of the bacteria. When it reacts with bacteria, triclosan forms a stable ternary complex by interacting with amino acid residues of the enzyme active site and inhibits the enzyme involved in the lipid biosynthesis pathway.^{17,29,30} It requires the uptake of triclosan by the bacteria, an unlikely scenario if triclosan is immobilized on PE. Therefore, the surfaces that show antimicrobial properties at low cell concentrations do not exhibit antibacterial effects in the high cell concentration suspension.

Figure 3 shows that before 20 h in the *E. coli* solution, the amount of viable bacteria on them is higher than that on sample 1 (control), although samples 2 and 3 show antibacterial effects and the bacteria biofilm has not formed. This phenomenon can be explained by the better hydrophilicity on samples 2 and 3 and easier adhesion of hydrophilic *E. coli* (Table I). After 20 h, the amount of bacteria on the modified samples continues to be more than that on the control sample, as the formation of the biofilm on the modified sample is easier. In contrast, Figure 4 indicates a

Table II Antimicrobial Performances of Modified Samples Against *S. aureus* and *E. coli*¹⁰

Samples	<i>S. aureus</i>	<i>E. coli</i>
Sample 2 (%)	99.1	99.9
Sample 3 (%)	96.2	94.7

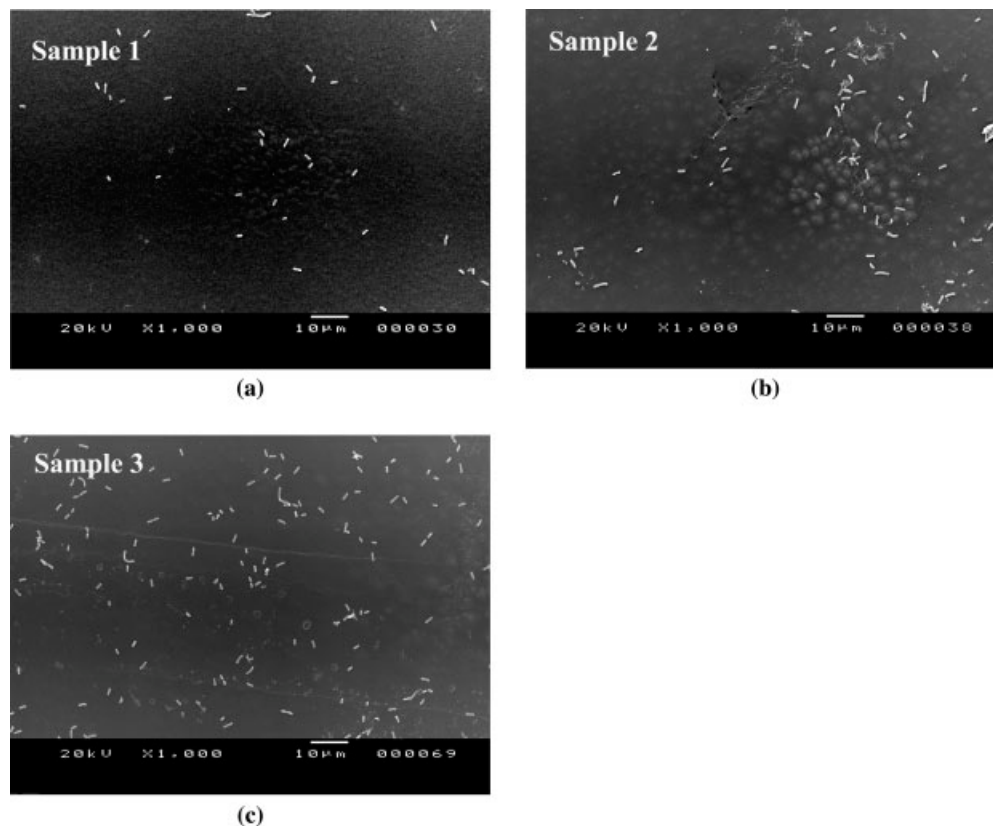


FIGURE 5 SEM images of *E. coli* adhered to the samples after exposure in the high cell suspension with about 10^8 CFU/mL for 3 h.

larger amount of *S. aureus* adhering onto the control sample compared to samples 2 and 3. This is because *S. aureus* adheres more effectively on a more hydrophobic surface. After incubation for 20 h, the results are consistent with those in Figure 3, and the amount of bacteria is significantly larger than that on the control sample.

SEM is often used to observe the biofilm formation on surfaces.^{19,32} In our experiments, the samples are monitored by SEM after soaking in the high bacteria concentration solution (10^8 CFU/mL) for 3 h. Figures 5 and 6 show the *E. coli* and *S. aureus* on the samples. The amounts of *E. coli* on the triclosan- and bronopol-modified PE are larger than that on the control sample. On the other hand, the amounts of *S. aureus* on the triclosan- and bronopol-modified PE are less than that on the control sample. The results confirm our explanation about bacteria adhesion on surfaces.

Bacteria Adherence at Low Cell Concentration

Based on our results, the antibacterial samples do not inhibit the formation of the bacteria biofilm in the

high concentration ($1 \sim 2 \times 10^8$ CFU/mL) suspension. However, as shown in Figures 7 and 8, the two kinds of bacteria at the lower concentration ($1 \sim 5 \times 10^6$ CFU/mL) exhibit a smaller degree of adherence on samples 2 and 3 than on sample 1. Bacteria adhesion on samples 2 and 3 increases initially and then decreases with time, eventually reaching a low level. This is because the modified sample surfaces have better hydrophilicity and are rougher, leading to stronger interactions between the bacteria and surfaces.^{26,27} The triclosan and bronopol immobilized on the surfaces kill or inhibit bacteria by affecting the membrane structure and forming a stable ternary complex via the interaction with amino acid residues at the enzyme active sites,^{17,29,30,33} but they cannot kill all the adhered bacteria immediately. Hence, the amount of adhered bacteria increases at the beginning. As soon as the bacteria are killed, the amount of adhered viable bacteria begins to diminish.

Figures 7 and 8 illustrate that *S. aureus* can more easily adhere onto the sample surfaces than *E. coli*, and the results are consistent with those described in the previous sections. The modified samples can inhibit a range of bacteria concentrations up to 10^6 CFU/mL.

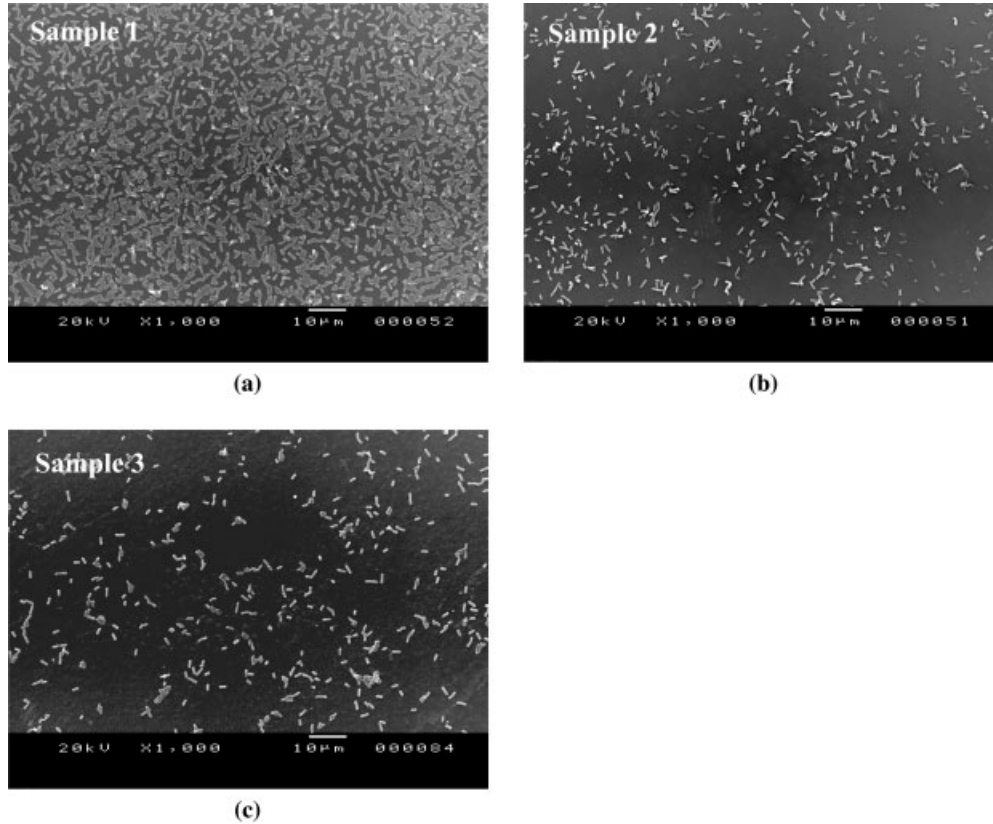


FIGURE 6 SEM images of *S. aureus* adhered to the samples after exposure to the cell suspension with about 10^8 CFU/mL for 3 h.

However, when the concentration exceeds $1 \sim 2 \times 10^8$ CFU/mL, they fail to inhibit biofilm formation. It should be noted that there are very few instances in biomedical applications where the bacteria concentrations exceed 10^6 CFU/mL and so these plasma-treated PE materials are very useful in practice.

CONCLUSION

Plasma immersion ion implantation is an excellent approach to enhance the antimicrobial properties of medical polyethylene. The modified PE surfaces not only have excellent antibacterial properties, but also

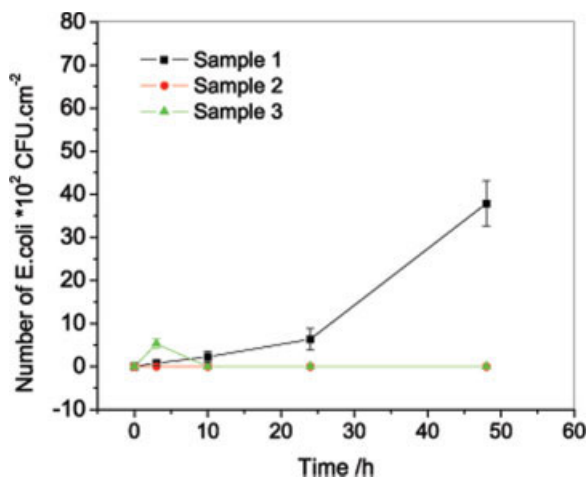


FIGURE 7 Adhered *E. coli* on the samples in low cell concentration suspension of $1 \sim 5 \times 10^6$ CFU/mL.

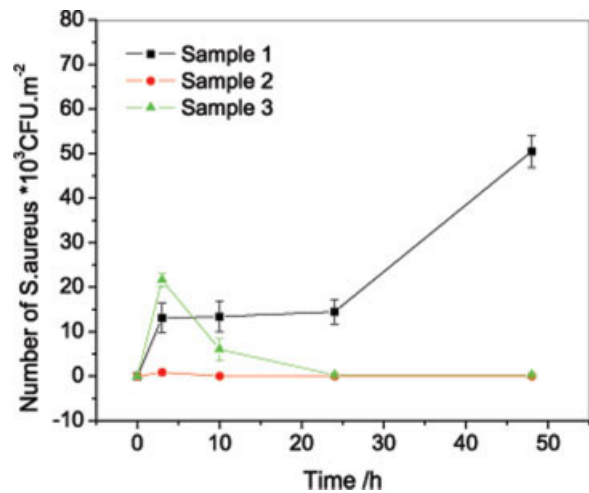


FIGURE 8 Adhered *S. aureus* on the samples in low cell concentration suspension of $1 \sim 5 \times 10^6$ CFU/mL.

exhibit remarkable bactericidal action even when placed in a suspension with a cell concentration of 10^6 CFU/mL. When the cell concentration goes up to 10^8 CFU/mL, they fail to develop noticeable resistance. However, in practice, resistance at this level is sufficient. The method described here is a relatively simple one that is useful in many biomedical and antibacterial applications.

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