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Synergistic antimicrobial effect against early biofilm formation: micropatterned surface plus antibiotic treatment

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ABSTRACT

The detrimental effects of biofilms are a cause of great concern in medical, industrial and environmental areas. In this study, we proposed a novel eradication strategy consisting of the combined use of micropatterned surfaces and antibiotics on biofilms to reduce the rate of bacterial colonisation. *Pseudomonas fluorescens* biofilms were used to perform a comparative evaluation of possible strategies to eradicate these biological layers. First, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration of planktonic cultures were determined. Subsequently, adhesion of bacteria on microstructured gold surfaces (MS) with patterned features that were similar to the bacterial diameter as well as on smooth nanostructured gold (NS) was assessed. As expected, lower bacterial attachment as well as inhibition of bacterial aggregation were observed on MS. The effect of streptomycin treatment (ST) in the concentration range 1–4 mg/L (0.25–1× MIC) on biofilms grown on MS and NS was also evaluated. The combined strategy involving the use of micropatterned surfaces and antibiotic treatment (MS + ST) to eradicate *Pseudomonas* biofilms was then investigated. Results showed a synergistic effect of MS + ST that yielded a reduction of ≥1000-fold in the number of surviving biofilm bacteria with respect to those obtained with single ST or MS. The combined strategy may be a significant contribution to the eradication of biofilms from different environments. In addition, the important role of early monolayer bacterial aggregates in increasing resistance to antimicrobial agents was demonstrated.

1. Introduction

Biofilm formation is a cause of great concern in relation to several areas of medical, industrial and environmental interest [1–3]. Biofilm is a lifestyle of several bacteria consisting of dense microbial communities attached to a substrate and embedded in an exopolysaccharide matrix. Its complex microstructure is a survival mechanism of microorganisms that protects them from aggressive agents in the environment. In fact, it is well known that to inhibit or eradicate biofilm bacteria, the minimum inhibitory concentration (MIC) of biocides greatly exceeds the concentration required to eradicate planktonic cells [1].

The initial step in the development of the biological layer (biofilm) is adherence of planktonic cells to a substrate [1,3,4]. Motile bacteria are able to spread easily on surfaces. Among motile bacteria, *Pseudomonas* spp. are frequently used as a model species to study bacterial adhesion and surface colonisation [5,6]. Following the initial attachment, bacteria form two-dimensional (2D) assemblages on the surface that move jointly in a co-operative way known as 'swarming motility'.

Several swarming bacteria such as *Pseudomonas* exhibit common survival strategies against antibiotics [7]. The biofilm plays important resistive roles, including: protection of the inner cells from the aggressive environment and, in the case of prosthesis, from the host immune defence system; creation of a diffusion barrier for large molecules, or sequestration of antibacterial substances; and reduction of the metabolic rate and induction of oxygen gradients throughout the biofilm that contribute to generating phenotypic heterogeneity within the bacterial population [1]. This adaptive resistance leads to antibiotic tolerance up to 1000-fold higher than for planktonic cells. Interestingly, the rate of mutation of *Pseudomonas* growing in biofilms is significantly higher than in planktonic bacteria. These factors explain the difficulty in controlling biofilm-related infection by simple conventional systemic antibiotic therapies [8]. Bacterial strategies also involve maintaining high cell density, minimising their exposed area to the harmful environment, and the sacrifice of microbes that are directly exposed to the antimicrobial agent [9]. Therefore, an effective

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strategy to improve antimicrobial action should impede the formation of compact 2D or 3D layers of bacteria.

Pseudomonas can be found ubiquitously causing severe problems in heterogeneous environments. Strains of *Pseudomonas fluorescens* have been frequently identified as inhabitants of soil, water and plant surfaces and transiently of the skin of humans. They deserve particular attention because they are involved both in detrimental and beneficial processes. The activity of *P. fluorescens* as a biocontrol species is beneficial in soil ecosystems (in plant growth or biodegradation promotion, biofertilisation) [10] and also in silk industry effluents, biodegradation of pollutants, biosurfactant production, etc. Among their deleterious effects, *P. fluorescens* have been implicated in urinary tract and bloodstream infections as well as procedure-related infections in hospitalised patients, particularly those with compromised immune systems [11–14]. In addition, these bacteria are also common sources of food contamination [15,16]. Owing to the detrimental consequences of *P. fluorescens* biofilm formation, strategies to kill these bacteria are of interest and a matter that deserves investigation.

Our previous studies [17–19] demonstrated that swarming microorganisms respond in similar ways to the same topography on surfaces that are chemically very different. We also reported that when the surface pattern of the microstructured surface is similar to the microbial size, many bacteria are trapped within the microfeatures of the topography and the isolation of single cells is promoted. In this study, we hypothesised that antibiotic action against these individual cells may be maximised if the gathering of attached cells is impeded. On the basis of these previous observations, a reasonable speculation is that a suitable combination of surface traps on the substrate and antibiotic treatment could act synergistically as an effective strategy to eradicate biofilms. To test this prediction, the effect of a combined strategy involving the use of micropatterned surfaces and antibiotics to reduce the rate of *P. fluorescens* colonisation was investigated.

2. Materials and methods

2.1. Substrates

Microstructured patterned inert surfaces (MS), consisting of a grid of 550 nm wide gold rows separated by 750 nm wide and 120 nm deep channels, were prepared by moulding and replication techniques as described previously [17–19]. As a control, nanostructured gold (NS) substrates having randomly oriented grains of 50–100 nm in size were used (Arrandee, Werther, Germany).

2.2. Bacterial cultures and biofilm formation

A *P. fluorescens* strain was grown on nutrient agar (Merck, Darmstadt, Germany) at 37 °C. Bacterial inocula were prepared in 300 mL of nutrient broth (Merck) by inoculating a loopful of cells from the nutrient agar plate and culturing overnight at 28 °C on a rotary shaker (250 rpm).

Afterwards, the cell suspension was adjusted to 10^8 colony-forming units (CFU)/mL in fresh growth medium and was used immediately for the inoculation of surfaces. The CFU was confirmed by viable count. The NS and MS substrates (area 0.25 cm^2) were placed in 24-well culture plates and 20 μL of bacterial suspension was seeded onto each substrate for 2 h at 37 °C to allow bacterial adhesion [20–22]. The substrates with biofilms were then removed and were washed gently by immersing in double-distilled sterile water in order to remove or detach those cells that were not tightly attached to the surface.

The number of bacteria adhered to the surface of the substrates was determined through quantification by the serial dilution method and plate counting. First, NS and MS substrates were individually placed in glass tubes containing 2 mL of sterile phosphate-buffered saline (PBS) and the irreversibly adherent bacteria were detached by sonication for 15 min with a Testlab sonicator (Testlab SRL, Bernal Oeste, Buenos Aires, Argentina). The number of bacteria in the sonicated suspension was then determined by serial dilution followed by bacterial culture on nutrient agar. A triplicate series of experiments and two replicates were carried out in each case.

2.3. Antibiotic treatment

The effect of antibiotic treatment was first evaluated on planktonic cells. The MIC of streptomycin against *P. fluorescens* was determined by the microtitre method as described in the Clinical and Laboratory Standards Institute (CLSI) guidelines [23] but replacing Mueller–Hinton broth by nutrient broth. The assay was performed in triplicate from independent bacterial cultures. The minimum bactericidal concentration (MBC) of streptomycin for *P. fluorescens* was also determined [24].

To test the combined effect of antibiotic treatment plus surface structure, NS and MS substrates were each exposed to a *P. fluorescens* culture for 2 h at 37 °C in 24-well culture plates as described in Section 2.2. Biofilms formed over the substrates were gently washed twice with PBS and were then incubated with 2 mL of serial two-fold dilutions ranging from 1 mg/L to 4 mg/L streptomycin in nutrient broth at 37 °C [streptomycin treatment (ST)]. After 18 h of incubation, the streptomycin solution was removed and the biofilms were washed twice with PBS solution. Subsequently, the substrates with biofilms were individually placed in glass tubes containing 2 mL of sterile PBS buffer and then sonicated. Aliquots of the resulting solution were plated, after appropriate dilution, onto nutrient agar plates. After 24 h of growth, colonies grown on the plates were enumerated. The same procedure was employed to obtain the number of viable cells attached on each substrate prior to antibiotic exposure. These values were used as control values to which the reduction of viable cells following antimicrobial treatment was referred. A triplicate series of experiments and two replicates were carried out in all cases.

2.4. Viability assays

After the incubation period, the substrates with biofilms were rinsed with 0.9% (w/v) NaCl solution and were dyed using the LIVE/DEAD *Bac*Light™ Viability Kit (Invitrogen, Carlsbad, CA) to evaluate the number of attached bacteria and dead cells.

The LIVE/DEAD stain was prepared by mixing 30 μL of staining component A (SYTO® 9) and 30 μL of staining component B [propidium iodide (PI)] and diluting the mixture to 1/200 in distilled water. Then, 6 μL of the dye was poured on each substrate and they were kept in the dark for 15 min at room temperature, after which the substrates were rinsed with sterile water. Fluorescent bacteria were visualised by epifluorescence with an Olympus BX 51 microscope (Olympus America Inc., Center Valley, PA). The microscope filters used were U-MWG2 (excitation 510–550 nm and emission 590 nm) and U-MWB2 (excitation 460–490 nm and emission 520 nm).

Bacterial viability was calculated from the ratio of the number of intact cells stained with SYTO 9 to the total number of cells [intact cells + PI-positive (damaged) cells]. Measurements were made using Image J software (National Institutes of Health, Bethesda, MD) for at least 10 randomly selected images.

2.5. Atomic force microscopy (AFM) and epifluorescence microscopy characterisation

Tapping® and contact mode AFM (Nanoscope V; Bruker, Santa Barbara, CA) were used to characterise the substrates using silicon tips (Arrow™ NCR; NanoWorld, Neuchâtel, Switzerland) (spring constant, 42 N/m; resonance frequency, 285 kHz) and silicon nitride tips (Bruker) (spring constant, 0.12 N/m), respectively. AFM was also used to image early stages of biofilm formation at the nanometre scale. In all cases, the topographic mode was used to image the samples. The roughness of the cell wall was calculated as the mean of 10 images using Nanoscope 7.30 software (Bruker).

To investigate global colonisation of the substrate surface, cells were dyed with acridine orange and were imaged using an epifluorescence microscopy technique. The samples were rinsed in double-distilled sterile water and were stained for 5 min with the fluorescent nucleic acid stain acridine orange at a concentration of 0.02% (w/v). Samples were then rinsed with double-distilled sterile water to remove excess stain. Microbial cells attached to the substrate were imaged using an optical microscope (Olympus BX 51) with the WB 2# filter cube in the light path (excitation filter BP 450–480 nm). The percentage of coverage was measured using Image J software.

2.6. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) to evaluate differences between groups. A *P*-value of <0.05 was considered statistically significant.

3. Results

To find a suitable strategy to reduce antimicrobial resistance, the effects of MS and ST as individual factors as well as the combined treatment (MS + ST) were evaluated.

3.1. Effect of microstructure as a single factor

The number of bacteria attached to the NS and MS surfaces after 2 h of colonisation was evaluated. In agreement with previous results [18], it was found that on the MS substrate the mean viable counts recovered was decreased significantly (>6-fold) compared with the NS substrate ($P = 0.006$) (Fig. 1a).

Epifluorescence and AFM images were taken in order to characterise the organisation of cells on both substrates (Fig. 1b–e). Fig. 1b and c depict the density of bacteria forming the biofilm after staining both substrates with acridine orange. Results show that $38.01 \pm 7.30\%$ of the NS area was covered by the biofilm, whilst only $10.03 \pm 6.20\%$ of the MS surface was covered by attached cells. As expected from previous results [17–19], AFM images revealed that most of the bacteria attached to the NS surface form open and ramified patterns (Fig. 1d), whilst on the MS substrate a large number of isolated bacteria were trapped at the channels and aligned with the channel axis following the direction of the pattern (Fig. 1e). Also, randomly distributed bacteria were seen on this surface. The lower density of cells on the MS substrate with respect to NS was evident (Fig. 1b and d compared with Fig. 1c and e). Epifluorescence microscopy and AFM images showed a good correlation with the results of bacterial counting on MS and NS surfaces.

3.2. Effect of antibiotic treatment as a single factor

The effect of ST was first determined on planktonic cells. MIC and MBC values for *P. fluorescens* were 4 mg/L and 8 mg/L, respectively.

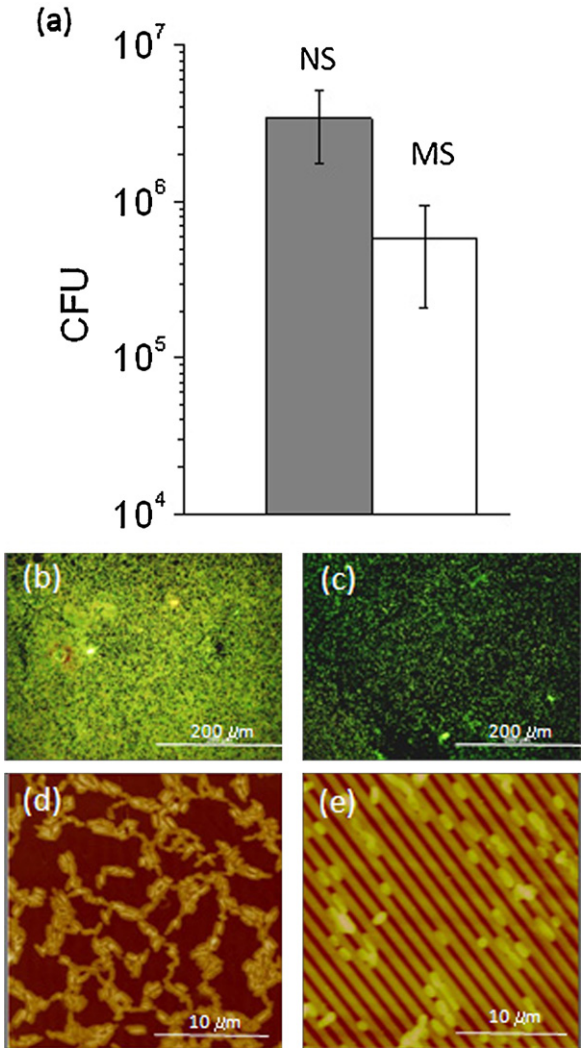

Fig. 1. (a) Number of *Pseudomonas fluorescens* bacteria attached to the smooth nanostructured gold (NS) and microstructured gold surfaces (MS) (area 0.25 cm^2) after 2 h of colonisation. (b and c) Epifluorescence images of *P. fluorescens* biofilm on NS (b) and MS (c) surfaces after 2 h of exposure to the bacterial culture. (d and e) Atomic force microscopy images of *P. fluorescens* biofilm on the NS surface (d) and *P. fluorescens* bacteria attached to the MS surface (e). CFU, colony-forming units.

Subsequently, the antimicrobial activity of streptomycin against a sessile population (2-h-old biofilms) formed on NS and MS substrates was analysed.

Fig. 2a shows the bactericidal effect of streptomycin against 2-h-old biofilms of *P. fluorescens* at concentrations ranging from 0.25× MIC to 1× MIC (1, 2 and 4 mg/L streptomycin) on the NS surface (single ST). Although the results of the number of viable bacteria attached to the NS surface did not differ significantly ($P > 0.05$) in the range of antibiotic concentrations tested, the number of cells attached on NS decreased up to 100-fold in comparison with those attached to the control (biofilmed-NS without ST).

3.3. Effect of the combined treatment

In contrast to the results obtained after the single antimicrobial treatment on biofilmed-NS (single ST), biofilmed-MS substrates showed a significant reduction in the number of sessile cells after addition of antibiotic (MS + ST). In fact, the number of bacteria attached on MS and treated with 2 mg/L streptomycin antimicrobial (MS + ST) decreased ca. 1000-fold with respect to the control and 1 order compared with the biofilmed-NS (single ST) ($P < 0.05$).

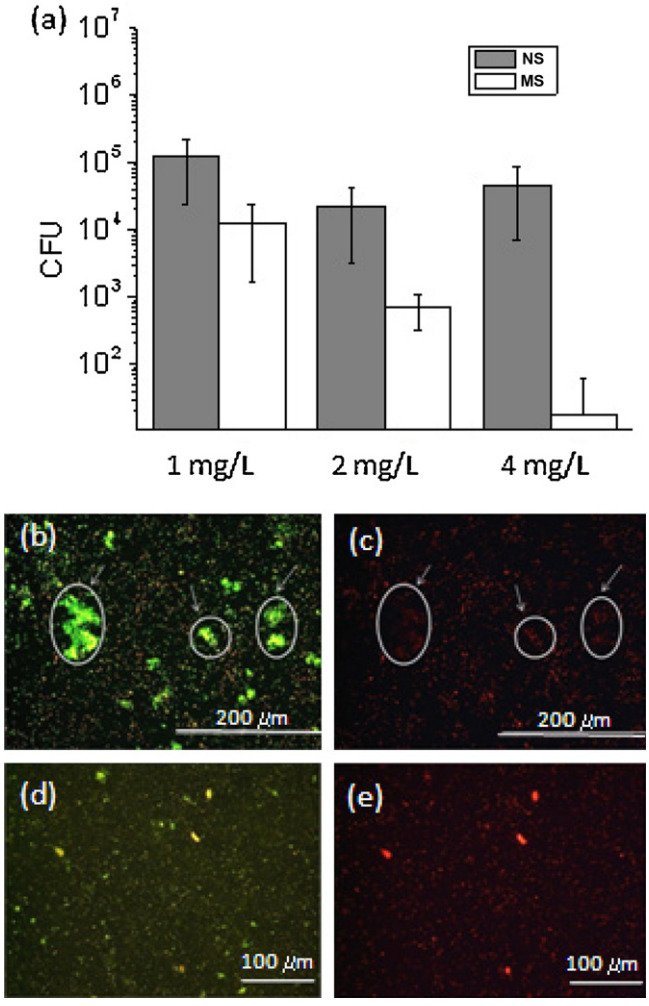

Fig. 2. (a) Number of viable bacteria after streptomycin treatment (ST) on nanostructured gold (NS) and microstructured gold surfaces (MS) (area 0.25 cm^2). (b–e) Epifluorescence image (LIVE/DEAD *Bac*Light™ Viability Kit) of *Pseudomonas fluorescens* on NS (b and c) and MS (d and e) after 2 h of ST. Green, live bacteria; red, dead bacteria. CFU, colony-forming units. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

After the combined treatment (MS + ST) with 4 mg/L streptomycin, a 10^5-fold reduction in the number of cells attached on MS with respect to the control (without ST) and 1000-fold compared with biofilmed-NS (single ST) was observed ($P < 0.05$).

AFM and epifluorescence microscopy (LIVE/DEAD kit) images were in accordance with the results of cell enumeration (Figs. 2 and 3). When sessile bacteria were exposed to streptomycin, epifluorescence images showed a higher bacterial density on NS (single ST) (Fig. 2b and c) than on MS surfaces (MS + ST) (Fig. 2d and e), with higher numbers of dead cells on the MS substrate (compare Fig. 2c and Fig. 2e). Importantly, compact bacterial microcolonies were observed on NS surfaces and a low number of dead cells were detected in these areas (compare areas limited by oval lines in Fig. 2b and c). However, these types of bacterial aggregates were not very frequent on MS, which exhibited the highest killing effect for the combined treatment (MS + ST, 4 mg/L) against sessile cells attached to the MS surface (Fig. 2e).

Interestingly, when bacterial surfaces were compared after ST, an important change in the membrane structure was evidenced in the case of bacteria attached to MS substrates. To evaluate this change, the average roughness (w) of the bacterial surfaces was calculated as:

$$w = \sum_{i=1}^{n} \left[\frac{(z_i - \bar{z})^2}{N} \right]^{1/2} \quad (1)$$

where N is the number of points considered on the surface, z_i is the height of point i on the surface and $\bar{z}$ is the average height of the N points. The average roughness corresponding to both surfaces with and without antibiotic treatment calculated according to Eq. (1) is shown in Table 1. The roughness of the bacterial surface attached to MS substrates after antibiotic treatment was twice that measured without antibiotics for MS. These findings can be appreciated in AFM images shown in Fig. 3. In the case of bacteria attached to NS substrates, no significant changes in the roughness of the cell wall were observed after ST.

Table 1
Roughness of cell membranes of *Pseudomonas fluorescens* attached both on NS and MS substrates without and with antibiotic treatment (0.5 mg/L).

	NS		MS	
	without ST	with ST	without ST	with ST
Roughness (nm)	7.19 ± 0.67	7.35 ± 2.43	9.41 ± 1.37	18.97 ± 1.76
P-value	0.918		0.002	

4. Discussion

On the basis of analysis of the characteristics of biofilms, it has been inferred that the dense structure of swarming bacterial aggregates is one of the main causes of biofilm resistance to antibiotic therapy [9]. A suitable strategy to reduce antimicrobial resistance may be based on hindering this bacterial aggregation. The effect of MS and ST as individual factors as well as combined treatment (MS + ST) were evaluated to find possible additive or synergic effects.

4.1. Effect of microstructure and antibiotic treatment as single factors

Analysis of AFM images revealed that the density of cells on the submicrometer-structured substrate (MS) was 6-fold lower than on the randomly nanostructured NS substrate; this implies an interesting effect of MS as single factor.

The other single factor evaluated was antibiotic treatment with streptomycin. In this case, the number of bacteria attached on NS was 100-fold lower than on the control (NS without ST). Results are in agreement with those previously reported that demonstrated the effect of engineering gold surfaces with patterns in the micrometre range in affecting the adhesion and motility of *P. fluorescens* aggregates [17].

4.2. Effect of combined treatment

The effect of the MS + ST combination was interpreted following Bonapace's criteria [25]. Accordingly, synergy in antimicrobial effect was defined as a ≥100-fold decrease in the number of bacteria after treatment with the combination MS + ST compared with the most active factor (ST or MS) and a ≥100-fold decrease with respect to the initial inoculum. On the other hand, additivity is ascribed to any other situation better than the most active factor but lower than a 100-fold decrease in bacterial number. Thus, these results indicated that the MS + ST combination exhibited synergy at 4 mg/L and additivity at 2 mg/L streptomycin. This confirms that the

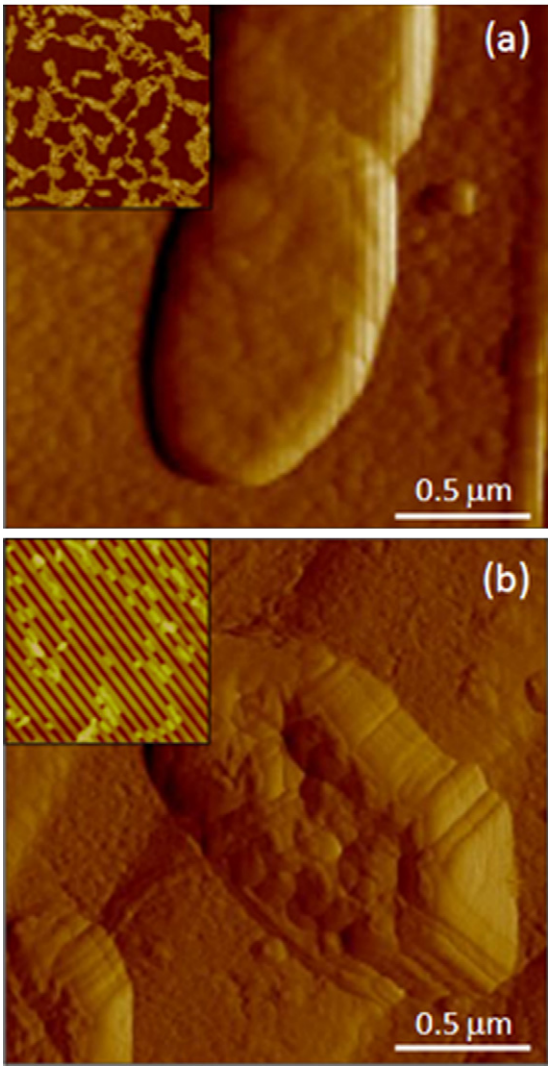

Fig. 3. Atomic force microscopy images (contact mode, deflection error) of the cell wall of bacteria attached on (a) nanostructured gold (NS) surface and (b) microstructured gold surfaces (MS) after streptomycin treatment. The insets show bacterial distribution on the substrates at lower magnification.

proposed strategy (MS + ST) could be effective and that it improved the bactericidal activity of streptomycin.

It is well known that antimicrobials cause morphological (size and shape) and surface (roughness, disruption) alterations in bacteria. The degree of this damage depends on the concentration and incubation time of antimicrobial treatment [26]. Related to this, changes in the roughness of the cell surface as a consequence of exposure to different concentrations of antibiotic [27] and other biocides [27,28] have been reported. In the present study, the increase in roughness of the cell wall was only observed on bacteria attached to MS substrates, whilst on NS the cell walls remain almost unaltered (Fig. 3). We speculate that on MS surfaces, where aggregate formation was hindered, there was a greater diffusion of antibiotics into the isolated cells than in the case of NS where the accumulation of bacteria in aggregates resulted in greater protection against the aggressive agent, reducing the antibiotic action. To the best of our knowledge, this is the first report of topological changes in the bacterial wall as a consequence of the action of an aminoglycoside antibiotic. This preliminary result will be studied in detail in the near future.

4.3. Role of monolayer biofilms in bacterial resistance to antimicrobial agents

As mentioned above, biofilms are considered to be highly resistant to antimicrobial agents. The mechanisms accounting for this resistance are multifactorial but the most notable are: limitation of antibiotic diffusion through the biofilm; slow growth owing to nutrient limitation; expression of genes involved in the general stress response; and emergence of the 'persister' cell phenotype. Since combinations of these factors are involved in most biofilm studies, it is still difficult to understand fully the mechanisms of biofilm resistance to antibiotics [29]. In this work, we focus on the antibiotic susceptibility of *P. fluorescens* cells growing in 2D or monolayer biofilms. This allowed exclusion of the effects of the restricted penetration of antimicrobial agents into multilayer biofilms as well as slower growth owing to nutrient limitation in the inner layer of the biofilm.

Several studies have reported that the susceptibility of a young adherent monolayer to antimicrobial agents is significantly higher than that of mature biofilms after 24 h of antimicrobial treatment [29,30]. However, differences in the susceptibility of monolayer aggregates in relation to attached isolated cells have not been clearly established. In this respect, the results of the current study demonstrated that after 2 h of incubation, formation of 2D aggregates was inhibited on MS, where a large amount of isolated cells were found. The antimicrobial effect of streptomycin on *P. fluorescens* monolayer biofilms formed on NS and on bacteria attached to MS (from the same amount of planktonic cells, i.e. 20 μL of 10^8 CFU/mL) is extremely different. MS biofilms showed a decrease of 10^5-fold in the number of living cells with respect to the control after being treated with 4 mg/L streptomycin, whilst a <100-fold decrease was found on NS biofilms after similar treatment. The question that immediately arises is why there was a synergetic bactericidal action against sessile cells when MS + ST are combined. To answer this question, it should be taken into account that we have previously reported [17] important changes in the shape and length of bacteria on MS. In fact, the length of a single attached bacteria on the trenches of the MS surface (1.44 ± 0.12 μm) was significantly shorter ($P < 0.01$) than the length of individual bacteria adhered to the NS surface (1.99 ± 0.12 μm, similar to those of planktonic cells). This reduction in bacterial size (MS effect) may be the result of unknown metabolic alterations that lead to an increase in the bactericidal activity of streptomycin (enhanced ST effect by MS). On the other hand, a microstructured surface yielded a 6-fold reduction in the number of attached bacteria (MS effect), many of which are trapped by the microfeatures. Under these conditions, motility was hindered and the gathering process was impeded [18], favouring the antibiotic action. Thus, adaptive resistance associated with high cell density within a 2D swarming colony was not achieved. In this respect, it must be taken into account that the action of aminoglycosides is dependent on their penetration of the cell wall to bind the ribosome and hence inhibit protein synthesis. This action is hindered when bacteria are gathered in 2D aggregates leaving less free exposed area for antibiotic penetration. This penetration is also obstructed by the polymeric substances that are produced by bacteria to stick to neighbouring cells of the aggregates. Instead, isolated cells on MS surfaces have almost the whole surface exposed to liquid medium and antibiotic action and consequently are more unprotected. Thus, the combined action of MS + ST was synergistic because the antimicrobial effect was exacerbated on surfaces with a lower amount of attached bacteria with a high percentage of isolated and phenotypically altered bacteria, which in turn made them more susceptible to antibiotic action.

This study is unique in that we explored the bactericidal effect of ST against sessile *P. fluorescens* cells when bacteria grow isolated or forming aggregates on gold surfaces without chemical

modification. In this sense, the important role of early 2D bacterial aggregates of *P. fluorescens* in increasing the resistance to antimicrobial agents was demonstrated.

The method proposed here is an antifouling strategy. Antifouling refers to a system that prevents the undesirable attachment and subsequent growth of organisms on a surface. This strategy is founded on a less toxic technology that prevents early biofilm growth and exacerbates the antimicrobial activity of antibiotics and biocides, reducing the use of these antimicrobial agents and consequently benefiting the environment. This scheme is potentially useful in several biomedical (countertops, doors, walls, beds, sanitary places, surgical tools, medical devices, drinking water systems, etc.) and industrial (pipes, filters, tubes, valves, storage vessels, desalination plants, cooling towers, ship hulls, etc.) areas. It may also show promise for environmental engineering applications that require the fine tuning of material interaction with microorganisms, including controlled bacterial immobilisation, antimicrobial properties and the ability to undergo surface modification.

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Ethical approval: Not required.

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