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The importance of 2D aggregates on the antimicrobial resistance of *Staphylococcus aureus* sessile bacteria

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ABSTRACT

Biofilms formed on implanted devices are difficult to eradicate. Adhesion mechanism, high bacterial density, aggregation, induction of persisters and stressed bacteria are some of the factors considered when the antimicrobial resistance of these biofilms is analyzed. The aim of this work was to provide an alternative approach to the understanding of this issue by using a specially designed experimental set up that includes the use of microstructured (MS) surfaces (potential inhibitors of bacterial aggregation) in combination with antimicrobial agents (streptomycin and levofloxacin) against *Staphylococcus aureus* attached cells. Biofilms formed on smooth surfaces were used as plain controls (biofilmed-PC) characterized by the formation of dense 2D bacterial aggregates. Results showed bacterial persistence when streptomycin or levofloxacin were applied to PC-biofilms. The antimicrobial activity of both antibiotics was enhanced when bacteria were attached on MS, where single cells or small aggregates were observed. Thus, dense 2D aggregates of bacteria seem to be crucial as a required previous stage to develop the antimicrobial resistance.

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1. Introduction

Implant-associated bacterial infections remain a serious complication in orthopedic surgery, with harmful effects on bone and soft surrounding tissues [1]. Among opportunistic human pathogen related to biomaterials *Staphylococcus aureus (S. aureus)* [2] are frequently isolated from metallic implants, while *Staphylococcus epidermidis* are commonly found on polymeric implants [3].

Biofilm formation is fundamental in the pathogenesis of biomaterials-associated infections [1,4]. The initial phase of biofilm growth is related to reversible attachment that is followed by an irreversible accumulative phase during which bacterial cells adhere to each other and grow to form a dense microbial community embedded in a self-produced exopolysaccharide matrix [5,6] with different architectures as well as dispersal mechanisms [7]. In clinical practice, despite the use of systemic antibiotic prophylactics and strict hygienic protocols the possibility exists that prostheses become contaminated with bacterial biofilms [4,6]. Biofilms are difficult to eradicate because their complex microstructure is a survival mechanism of microorganisms that protects them from antimicrobial agents, phagocytosis and other components of the innate and adaptive immune and inflammatory defense system of the host [8]. This adaptive resistance of biofilm leads to antibiotic tolerance up to 1000-fold higher than non-aggregated planktonic cells. Thus, systemic antibiotics are able to eliminate planktonic cells and bacteria released from the biofilm but fail in killing embedded bacteria.

In the last decades much attention has been paid to the growth of the multilayer (3D) biofilms. However, the adhesive steps that mediate transient to permanent surface attachment, including a two dimensional (2D) monolayer formation, are crucial to understand the progress of biofilm-related infections [7,9] and deserve more awareness. The monolayer biofilm is a single layer of cells adhered to a surface [10]. This type of configuration may be favored when cell–surface interactions predominate over cell–cell interactions [11–16]. There has been considerable interest in *Pseudomonas* 2D swarming layers but scarce information is available in relation to 2D layers of non-motile bacteria like *Staphylococcus* (of critical importance in implant-related infections). Therefore, a better understanding of the antimicrobial susceptibility of *S. aureus* in early biofilm development is required to apply an effective antibiotic therapy in hospitalized patients.

The aim of the study was to evaluate the role of cell adhesion and aggregation as well as the bacterial density on susceptibility of *S. aureus* biofilms to antimicrobial agents. We focused on the contribution of bacterial aggregation, as single factor, to the antibiotic resistance of sessile cells in the early stage of biofilms formation, i.e. in the phase of 2D aggregates formation on the surface. To this aim, we used a strategy developed in the lab, based on the use of microstructured surfaces (MS) to keep sessile cells isolated. MS was previously successfully tested on motile *Pseudomonas*

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fluorescens (P. fluorescens) preventing the bacterial aggregation and, thus, the formation of mature three dimensional (3D) biofilms. This strategy avoids the use of mutant strains (with alteration in the bacterial attachment and/or biofilm formation with pleiotropic effects) frequently employed to elude the formation of aggregates [17,18]. The antibiotic susceptibility of these isolated sessile cells (adherent cells, without bacterial aggregation) was compared with 2D biofilms (monolayer biofilm of aggregated cells) formed on planar surfaces (planar control). Therefore, this approach is unique because it allowed the evaluation of antimicrobial activity of antibiotics on isolated sessile cells (wild type strain), without the use of biofilm-defective mutant strains, as well as without mechanical separation techniques that may induce cell damage. It is worth to mention that sessile cells on MS are free or exhibited a minimum amount of extracellular polymeric substances (EPS). In this way, it is possible to investigate the antibiotic action against non-altered isolated and aggregated sessile cells. The response of biofilms of motile bacteria (*P. fluorescens*) to the antibiotic treatment (AT) was also included with the aim of comparison with that of non-motile *S. aureus*.

Two ATs with different mechanisms of action were used for comparison: levofloxacin and streptomycin. Levofloxacin is a fluoroquinolone drug class. Its broad spectrum includes Gram(+) bacteria such as *S. aureus* and *S. epidermidis* [19]. The mechanism of action of levofloxacin comprises the inhibition of bacterial enzymes required for DNA replication, transcription, repair, and recombination. On the other hand, streptomycin is a broad spectrum antibiotic whose mechanism of action, like other aminoglycosides, involves the inhibition of the protein synthesis that eventually leads to microbial death. The bactericidal or bacteriostatic effects of these two ATs on biofilms and attached single cells were compared under identical experimental conditions.

The role of persisters in the resistance of bacterial aggregates to AT was also analyzed. Persisters are phenotypic variants of wild bacteria which are genetically identical to susceptible bacteria. They are generally non-growing but their phenotypic tolerance allows them to remain viable in the presence of bactericidal antibiotics [20]. These particular bacteria seem to establish active defense systems towards oxidative stress imposed by bactericidal antibiotics using induced mechanisms. It has been suggested that persisters, rather than biofilm architecture, are responsible to resistance of biofilms to killing agents [21]. Persister cells are mixed with stressed cells and normal cell in the mature biofilm and consequently, the concentration required to kill all these stressed cells is higher than those corresponding to normal cells but lower than that needed to kill persisters. Both, biofilms and dense aggregates of planktonic cells can be sources of persisters. Detection of specific signaling molecules by quorum sensing, allowing bacteria to sense their cell density, probably plays an important role in relation to the induction of persisters [20]. Interestingly, upon removal of aggressive conditions, persisters switch back to their growing mode and consequently can be detected by plate counting [22].

2. Materials and methods

2.1. Substrates

Microstructured patterned inert surfaces (MS), were prepared by molding and replication techniques, as described elsewhere [23–25]. These MS surfaces consisted in a grid of 550 nm wide gold rows separated by 750 nm wide (close to bacterial diameter) and 120 nm deep channels. As control plain gold substrates (random nanostructured surface, PC), having randomly oriented grains of 50–100 nm in size and 8 nm in height were used (Arrandee®, Germany) [11,12,15]. Please, see supplementary data for further information.

2.2. Bacterial cultures and biofilm formation

S. aureus ATCC 25923 was grown in Nutrient Broth (Merck, Darmstadt, Germany) at 28 °C with shaking (250 rpm) overnight. Following incubation, the bacterial suspension was adjusted to 10^8 colony-forming units (CFU) mL^{-1} in fresh growth medium which was confirmed by viable count method.

The experiments of bacterial adhesion on MS and PC substrates were performed using the procedure previously described in the literature [26]. Briefly, the cell suspension (10^8 CFU mL^{-1}) was used for the inoculation of surfaces. The substrates (PC, MS, 0.25 cm^2) were placed in 24-well culture plates and 20 μl of bacterial suspension was deposited onto each substrate for 2 h at 37 °C to allow bacterial adhesion (2 h-adhesion). Then the substrates with sessile bacteria were kindly removed horizontally so that there was always a liquid column above the surface, and immersed in sterile phosphate buffered saline solution (PBS, 10 mM, pH = 7.3) in order to remove those cells which were not tightly attached to the surface.

The quantification of attached bacteria to the surface of the substrates was performed by serial dilution method and plate counting after their detachment by sonication. With this purpose, PC and MS substrates were individually placed in glass tubes containing 2 mL of sterile PBS and the irreversibly adherent bacteria were detached by sonication for 15 min with a Testlab ultra-sonic bath (40 Khz with power output of 160 W). Later, the number of bacteria in the sonicated suspension was 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. No effect of sonication on the number of surviving materials was found ($3.15 \times 10^7 \pm 1.63 \times 10^7$ CFU mL^{-1} without sonication and $3.75 \times 10^7 \pm 0.92 \times 10^7$ CFU mL^{-1} with sonication).

2.3. Antibiotic susceptibility of planktonic and sessile cells of *S. aureus*

Antibiotics susceptibility testing was firstly applied to planktonic cultures. The minimum inhibitory concentration (MIC) of streptomycin and levofloxacin against planktonic (P-MIC) *S. aureus* strain was determined by the microtiter method as described in CLSI guidelines [27] but replacing Müller–Hinton broth by nutrient broth. The P-MIC was defined as the lowest concentration of antibiotic where bacterial growth was not detected. The minimum bactericidal concentration (MBC) of planktonic cells (P-MBC) of streptomycin and levofloxacin for planktonic *S. aureus* was determined by plate count method [28]. The antibiotic concentration that produced 99.9% mortality was considered as the P-MBC. The assays were performed in triplicates from independent bacterial cultures.

To test the effect of ATs on isolated sessile cells (adhered cells, without bacterial aggregation) and aggregated bacteria, *S. aureus* biofilms were formed on MS (biofilmed-MS) and PC substrates (biofilmed-PC) for 2 h at 37 °C in 24-well culture plates as described in the previous section. Biofilms formed on the substrates after 2 h exposure to the bacterial culture were gently washed twice with PBS and then incubated with 2 mL of nutrient broth containing streptomycin or levofloxacin in serial two-fold dilutions for concentrations in the 1–4 μg/mL, and 0.25–1 μg/mL ranges, respectively. After 18 h of incubation at 37 °C, the antibiotic solutions were 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 and then sonicated. Aliquots of the resulting solution were plated, after appropriate dilutions, onto nutrient agar plates. After 24 h growth period 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 the exposure to each AT. These values were used as control values to which the reduction of viable cells after antimicrobial treatment was referred [29]. A triplicate series of experiments and two replicates were carried out in all cases.

2.4. Viability assays by epifluorescence microscopy

The initial colonization of *S. aureus* before AT and the number of attached bacteria (live and dead cells) after levofloxacin treatment (0.25 and 0.5 μg/mL) on the substrate surface, was determined by using LIVE/

DEAD BacLight ® viability kit (Invitrogen) and epifluorescence microscopy technique. 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) and diluting the mixture to 1/200 in distilled water. 6 μL of the dye was poured on each substrate and then they were kept in the dark for 15 min at room temperature. After that, the substrates were rinsed with sterile NaCl solution (0.9% *w/v*). In this case, phosphate wash buffer was not used because of its probable influence on staining process reducing its efficiency [30]. Fluorescent bacteria were visualized by epifluorescence with an Olympus BX-51 microscope. The microscope filters used were U-MWG2 (excitation 510–550 nm and emission 590 nm) and U-MWB2 (excitation 460–490 and emission 520). Bacteria were kept hydrated throughout the entire procedure.

Semi-quantitative analysis of the fractional bacterial coverage (%) on each surface was made from the epifluorescence images by measuring the total area covered by cells and referring the value to the area of the image. The percentage of the substrate area covered by bacteria was assumed as proportional to the total number of cells. The measurements were made by using Image J software for at least 10 randomly selected images.

2.5. Atomic force microscopy (AFM)

The samples were prepared as described in Section 2.2 and dried in air before imaging.

Ex-situ Tapping® and contact mode atomic force microscopy (AFM) (Nanoscope V, Bruker, Santa Barbara CA) was used to characterize the substrates using silicon tips (Nano World ® Arrow NCR, spring constant: 42 N/m, resonance frequency: 285 KHz) and silicon nitride tips (Buker, spring constant: 0.12 N/m), respectively. AFM (Tapping® mode) was also used to image early stages of biofilm formation at nanometer scale. In all cases the topographic mode was used to image the samples. In order to disregard any influence of dewetting forces on the location of bacteria, control experiments were done by imaging the samples by epifluorescence microscopy keeping the sample wet during the whole process. This leads to the conclusion that the organization of the cells on both surfaces was not affected by the drying of the sample.

2.6. Scanning electron microscopy (SEM) analysis

S. aureus biofilms formed on PC and MS substrates before and after levofloxacin treatment (1 μg/mL) were examined by Scanning electron microscopy (SEM) and prepared as described above. Preformed biofilm were washed with PBS and immersed into a fixing solution containing 2% glutaraldehyde and 0.1 M sodium cacodylate (NaCac) at pH 7.2, and kept for 2 h; after that, they were washed three times for 10 min with 0.1 M NaCac buffer. The samples were dehydrated in a gradient series of ethanol solutions (30, 50, 70, 90, 95, 100%). For analysis with scanning electron microscope, the biofilms were critical point dried and covered with a 20-nm layer of gold. SEM images were better than AFM to analyze the microstructure of 3D aggregates.

2.7. Statistical analysis

Statistical analysis was performed using one-way analysis of variables (ANOVA) to evaluate differences between groups. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Microstructured surfaces (MS) as inhibitor of S. aureus aggregation

In order to characterize the organization of *S. aureus* sessile cells on MS and planar substrates, AFM, SEM and epifluorescence microscopy were used (Fig. 1 a–d). Microscopy images revealed that the density of nonmotile bacteria attached on PC surfaces was higher than those of MS substrates, and the bacteria coverage was 57% and 11% on PC and MS, respectively.

Isolated cells and ditches are clearly imaged by AFM. However, rough features on surfaces, such as those formed by three dimensional (3D) bacterial aggregates on PC surfaces with AT (see Fig. 3) are hardly imaged by AFM. Thus, in order to analyze the cell adhesion on PC surfaces, SEM imaging was used. Fig. 1b shows that the biofilm grown on the PC substrates forms a dense 2D ramified pattern (2D network distribution). Control experiments keeping the samples wet (see supplementary data) demonstrated the absence of any influence of dewetting processes. The 2D aggregation was confirmed by AFM, measuring the height of the aggregates which was close to cell diameter in several sites (data not shown). Isolated bacteria adhered to MS substratum and trapped in the ditches could be detected by AFM. Colonization of PC and MS surfaces (2 h-biofilms) by *S. aureus* was assessed by plate counting. The numbers of sessile cells (CFU) attached on PC and MS surfaces are $4.44 \times 10^5 \pm 2.73 \times 10^5$ and $1.24 \times 10^5 \pm 9.20 \times 10^4$ UFC/mm^2 respectively, with similar relationships than previous results (Fig. 1). The number of viable bacteria in biofilms formed on smooth PC surfaces (PC-biofilms) was slightly higher than those of MS substrate. However, this difference is significant ($p = 0.007$).Notice that the rate between the number of viable bacteria (average) from plate counting on PC and MS substrate is 3.58 while the relation of bacteria coverage PC to MS substrate is 5.18. The difference could be explained taking into account that bacteria coverage include viable and dead bacteria adhered to the substrate while plate counting method only enumerate viable cells. Moreover biofilmed-PC exhibited aggregated bacteria surrounded by biofilm matrix (EPS) which could overestimate the number of bacteria on this surface.

3.2. Antibiotic susceptibilities of S. aureus planktonic cells and aggregated (biofilmed-PC) sessile cells

In vitro susceptibility of *S. aureus* to levofloxacin and streptomycin was firstly determined on planktonic cells. The P-MIC and P-MBC for *S. aureus* were 0.5 μg/mL and 1.0 μg/mL for levofloxacin, and 2 μg/mL and 4 μg/mL for streptomycin, respectively.

Subsequently, the bactericidal activity of levofloxacin and streptomycin against sessile cells (2 h-adhesion) formed on PC substrates was analyzed in 0.5× P-MIC–2× P-MIC concentration range. Fig. 2 depicts the antibacterial effect of levofloxacin and streptomycin against 2 h-biofilms of *S. aureus* on PC surface (PC-biofilms + AT). Results showed that levofloxacin could not inhibit the growth of aggregated sessile cells on PC at 0.5× P-MIC (0.25 μg/mL); ($p > 0.05$ compared with PC-biofilms without AT), but exhibited an inhibitory effect at 1× P-MIC (0.50 μg/mL). At 2× P-MIC (1 μg/mL) bactericidal activity (reduction of the number of bacteria by 3 log10 units after exposure to AT for 18 h) was observed. Thus, MIC and MBC values of levofloxacin for aggregated sessile bacteria were 0.5 μg/mL and 1 μg/mL respectively, coincident with P-MIC and P-MBC.

On the other hand, the viability of PC-biofilms decreased up to 100-fold when they were treated with streptomycin at concentrations ≥1× P-MIC (≥2 mg/ml, $p < 0.05$), in comparison to those initially attached to the PC surface, that is, when compared to the initial sessile cells on PC (2 h biofilmed-PC without AT).

However the number of viable bacteria attached to the PC surface after antibiotic addition did not differ significantly in the concentration range tested (0.5× P-MIC–2× P-MIC). Therefore levofloxacin exhibited inhibitory (1× P-MIC = 0.5 μg/mL) and bactericidal activity (2× P-MIC = 1 μg/mL), while streptomycin shows only inhibitory action in the 0.5–2× P-MIC concentration range against aggregated sessile cells on PC.

3.3. Antibiotic effect on isolated sessile cells of S. aureus (biofilmed-MS)

The effect of single 18 h antimicrobial treatment on isolated sessile cells (biofilmed-MS) showed a significant reduction in the number of

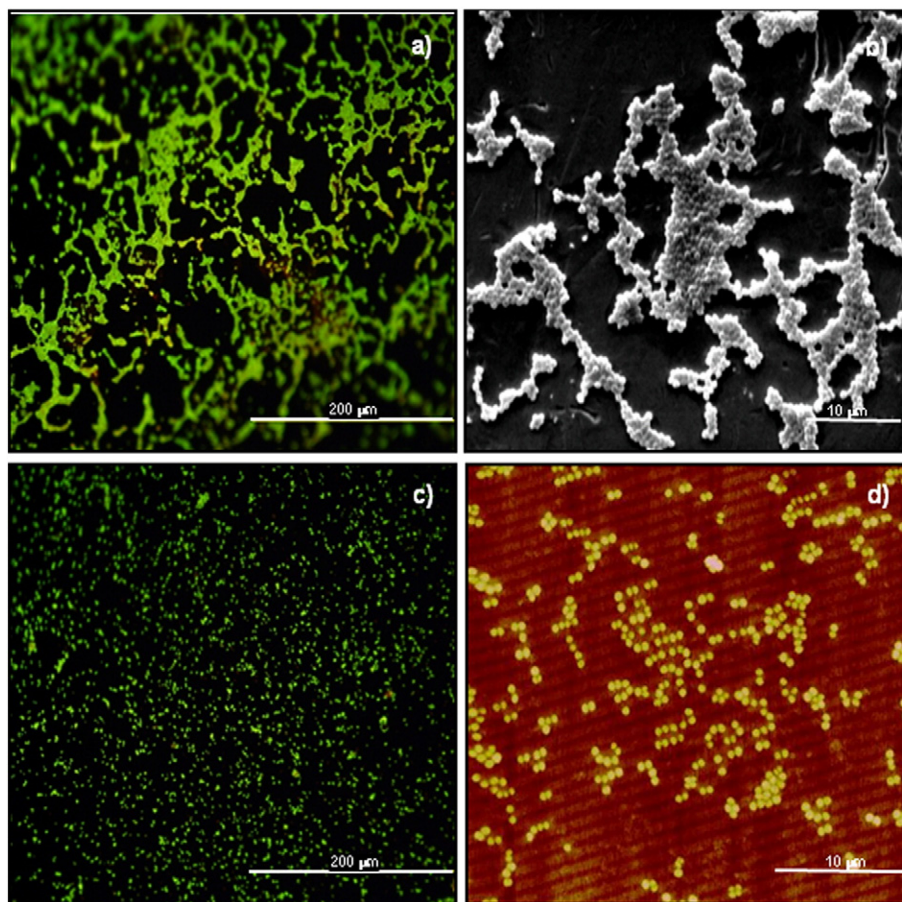

Fig. 1. Images of sessile *S. aureus* (2 h-biofilm) before AT on planar control (PC) gold surface (a, b) and on microstructured (MS) gold surfaces (c,d). a) Epifluorescence microscopy (BacLight Live/Dead viability kit; green, live bacteria; red, dead bacteria); b) SEM image; c) epifluorescence microscopy (BacLight Live/Dead viability kit; green, live bacteria; red, dead bacteria); d) AFM image. Substrate surface area: 25 mm^2.

viable bacteria after the antibiotic addition when compared to both, the initial CFU on MS and the viable cells on PC substrates after the same AT (Fig. 2). In fact, the number of bacteria attached on MS substrates and treated with 0.5× P-MIC of levofloxacin or streptomycin (biofilmed-MS + AT) decreased about 100-fold with respect to the initial biofilmed-MS.

On the other hand, the viability of isolated sessile cells on MS after treatments with 1× P-MIC values of levofloxacin or streptomycin, decreased by over 10^4-fold when compared with the initial PC-biofilms and 10^3-fold when compared with PC-biofilm + AT (1× P-MIC, $p < 0.05$). Moreover, results of cell enumeration at 2× P-MIC of levofloxacin or streptomycin showed that viable bacteria on MS decreased by over 10^5-fold with respect to those attached to the initial PC-biofilm without AT. Thus, the treatment with both antibiotics, levofloxacin and streptomycin, applied to isolated sessile cells, showed an excellent bactericidal activity within the range of 1× P-MIC to 2× P-MIC, and exhibited bacteriostatic effect at 0,5× P-MIC (cells viability decreased up to 64-fold with respect PC-biofilm + AT).

In order to image *S. aureus* biofilm after levofloxacin treatment at 1 μg/mL on both substrates, AFM and epifluorescence microscopy (Live/Dead kit) were used. As shown in Fig. 3, live/dead-stained biofilms showed small groups of red cells on MS substrate, indicanting that levofloxacin afected isolated sessile bacteria. In contrast, PC substrate exhibited mature *S. aureus* biofilm with predominant red sessile cells (green cells mostly in the inner side of biofilm), in concordance with cell enumeration. Formation of·3D biofilms (2 h biofilms + 18 h with AT) were clearly distinguished by SEM (Fig. 3b) on PC while isolated bacteria, some of them trapped on the ditches, can be very well imaged by AFM on MS.

The behavior of motile, Gram(−) *P. fluorescens* (data from Ref. [26]) and non motile, Gram(+) *S. aureus* growing on PC and treated with different concentration of streptomycin (concentration range 1–4 μg/mL) is compared in Fig. 4. The curves revealed that after an initial drop a further increase in the antibiotic concentration had weak effect on killing.

4. Discussion

Formation of biofilms on metal implants is one of the main causes that leads to failures of dental and orthopedic implants. Conventional AT is frequently not successful in impeding local infections. New strategies are needed to enhance the antibiotic effectiveness. With the aim of finding a new method to improve the antibacterial action the use modified surfaces to inhibit bacterial adhesion and enhance the AT killing action is proposed here. We hypothesized that 2D bacterial aggregates offer high resistance to AT and that AT action could be improved if isolation of attached bacteria was achieved. Isolated cells attached on surfaces were obtained using an innovative strategy. It consisted in the utilization of MS surfaces without employing either mutated *S. aureus* or mechanical treatments frequently used in literature for this purpose [31]. On this regard, we have previously reported that microstructured surfaces with characteristic dimensions similar to the diameter of bacteria are able to inhibit the aggregation of motile cells, avoiding the formation of raft-like structures necessary for their cooperative movement [32,33]. In agreement with these results, non motile *S. aureus* are mostly trapped within the channels of the MS substrate (Fig. 1d) probably due to the fact that the cells find a greater surface (bottom and walls of the channels) for the adhesion than on PC substrates. Thus, the effect of two antibiotics on isolated sessile cells can be evaluated in comparison to

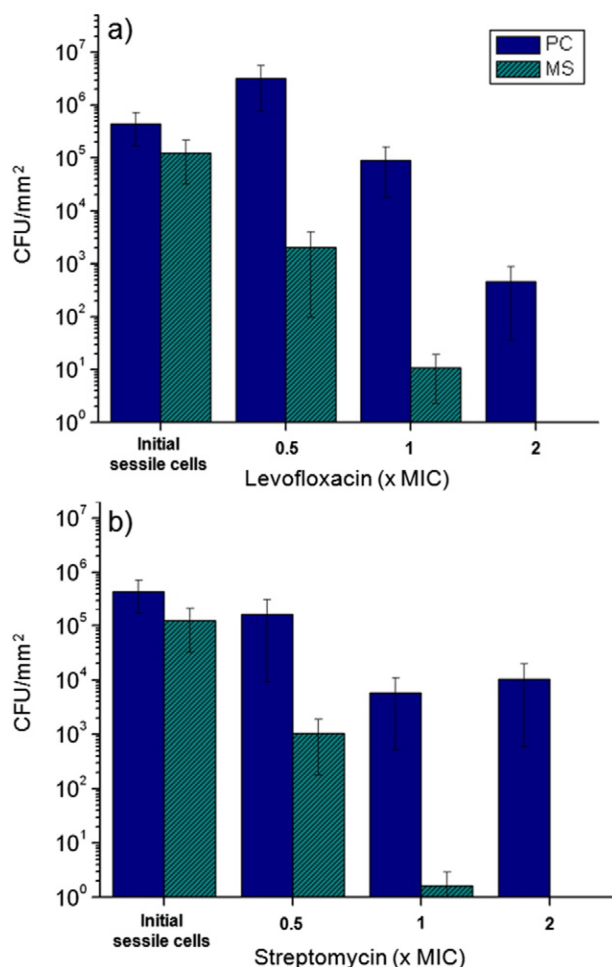

Fig. 2. Effect of antibiotic on viability of isolated sessile cells (MS) and 2D biofilms (PC). Results are expressed as mean values SD. Levofloxacin, LEV; streptomycin, STP. Initial sessile cells are the number of viable bacteria before the antibiotic treatment.

dense *S. aureus* 2D biofilms attached on solid surfaces of identical composition.

4.1. Antibiotic action against planktonic and sessile cells

ATs with levofloxacin and streptomycin exhibited excellent bactericidal activity on planktonic bacteria. With the aim of selecting the appropriate concentration range P-MIC and P-MBC values were determined and resulted in 0.5 and 2.0 mg/L, for levofloxacin and streptomycin, respectively with P-MBC = 2× P-MIC. Biofilm response showed markedly higher resistivity. Thus, in case of streptomycin about 10^4 cells/mm^2 remained viable at P-MBC concentrations.

Dramatic differences in AT efficacy against *S. aureus* growing on PC (Fig. 2) were found after treatment with 0.5× P-MIC: levofloxacin did not inhibited the growth of sessile cells, while streptomycin exhibited bacteriostatic effect. Interestingly, after AT with 2× P-MIC (P-MBC), levofloxacin showed bactericidal activity against biofilms while in case of streptomycin it was only bacteriostatic. Even though levofloxacin was more efficient in killing sessile cells, the initial 2D structures and small aggregates found on PC substrates were not broken up during the levofloxacin treatment (Fig. 3 a,b). Thus, the formation of mature biofilm after levofloxacin exposure at 2× P-MIC, and the presence of c.a. 5×10^2 CFU/mm^2 viable sessile cells (Fig. 2) are consequences of the intrinsic antibiotic resistance of biofilm and the failures in AT applied to medical devices [34].

Several mechanisms have been proposed to elucidate the most important factors leading to the biofilm resistance to ATs. The marked difference between sessile and planktonic cells has been attributed to unspecific alteration of the phenotype of sessile cells [35,36]. It was also found that bacterial population reveals different grades of resistance: normal cells, stressed cells (those with significant AT tolerance) and persister cells (those with very high AT tolerance: 1000–10,000 times higher than P-MBC) [37]. On this respect, microscopic observations (Fig. 3) revealed that streptomycin or levofloxacin treatments were unable to disrupt bacterial aggregates. The low efficacy of streptomycin was also reported by Kirby et al. [38] who investigated the susceptibility of biofilms to 8 antimicrobials including streptomycin and found that it was not effective in killing sessile cells but was successful in treating planktonic cells. This reduced killing action of streptomycin could be attributed to the presence of both, stressed and persister cells [37] within the assemblages formed during the AT period. Shapiro et al. [39] reported that *S. epidermidis* biofilms after levofloxacin treatment exhibited a high level of persistence, at least 9% of persister bacteria, showing that these cells have an important role in the survival mechanism of biofilms. Although many bacteria were killed the remaining bacteria were able to duplicate in the presence of streptomycin and hence form resistant 3D matrixes on PC.

The presence of persister cells can be inferred from survival curves since the number of viable bacteria remains unchanged as the antibiotic concentration increases [32,36,37]. The resistant behavior of both *P. fluorescens* and *S. aureus* growing on PC and treated with different concentration of streptomycin is shown in Fig. 4. From these results, it can be concluded that a high number of resistant cells (stressed + persister cells > 10^3 survivals/mm^2) may be induced after 18 h streptomycin treatments. Thus, in agreement with a previous report, present results demonstrated that streptomycin (like other aminoglycosides) display weak activity against *S. aureus* and encourage the generation of resistant cells among the biofilm formed on PC [41,42].

4.2. Antibiotic action against dense bacterial aggregates and single cells

Even though it is widely accepted that sessile cells are more resistant to antimicrobial treatment than planktonic cells the contribution of physical structure of microbial aggregates and cell density of both, planktonic and sessile cells, to the antibiotic susceptibility deserves investigation [8,14,38]. Haaber et al. [8] contrasted the response of planktonic aggregates to that of single planktonic cells. They found that aggregates were protected against the killing action of several antibiotics (erythromycin, vancomycin, kanamycin and ciprofloxacin) and survived significantly better. However, to our best knowledge, comparison between the susceptibility of single sessile cells and sessile aggregates has not been reported.

In order to analyze the influence of the grade of aggregation of sessile cells on streptomycin and levofloxacin action, the effect of AT on cells attached on PC surfaces (with sessile networks and large aggregates) and on MS surfaces (with isolated cells and small aggregates) was compared. Previous studies showed that MS surfaces inhibited the aggregation of motile bacteria with swarming abilities when the characteristic dimensions of the microstructural pattern were similar to the bacterial diameter [12,15,24,25].

Fig. 2 showed that, independently of the antibiotic nature, the efficacy of the AT was improved when sessile bacteria were isolated or within small aggregates on MS. Conversely, 2D networks (2 h monolayer biofilm) initially formed on PC surfaces (Fig. 1 a,b) were able to resist the antimicrobial action and grow forming 3D (multilayer) structures during the 18 h AT. Accordingly, it has been reported that monolayers were not less resistant to antibiotic challenge than multilayers [37].

Importantly, almost complete eradication of bacteria growing on MS surfaces was achieved using either 2× P-MIC streptomycin or levofloxacin while more than 5×10^2 cells/mm^2 remained alive on PC surfaces after similar treatments. Evidently single cells and small

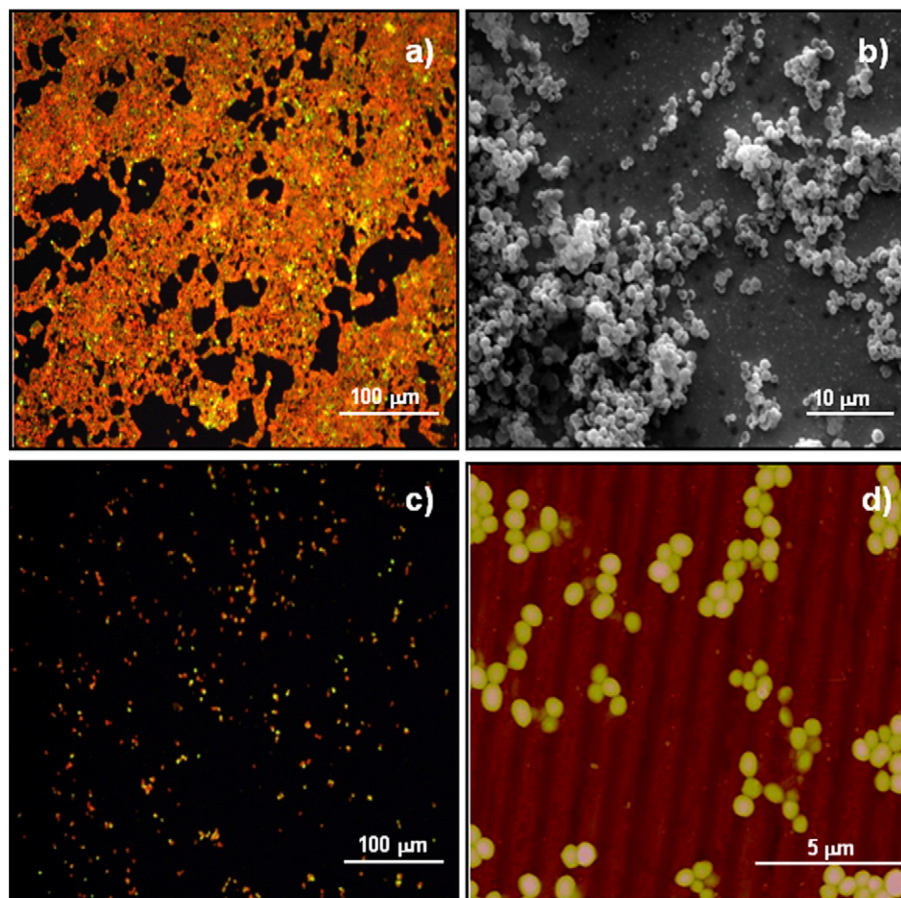

Fig. 3. Bacteria attached to PC (a, b) and MS (c, d) after 18 h of levofloxacin treatment (1 mg/L, P-MBC). (a) Epifluorescence image of 3D aggregates with dead cells (red) on the surface and live cells (green) in the inner layers on PC substrate; (b) bacteria on PC surface imaged by SEM; (c) epifluorescence image of small 2D assemblages and individual cells, many of which are dead, on MS surface; and (d) bacteria on MS surface imaged by AFM.

aggregates attached on MS surfaces exhibited higher susceptibility to ATs than the 2D networks with densely packed bacteria formed on PC.

Qu et al. [37] proposed that the presence of resistant cells, rather than the quantity of resistant cells in biofilms, may be the key for biofilm resistance. Thus, high density 2D young biofilms is probably as resistant as 3D mature biofilms. They and other authors suggested that the adherent growth mode rather than the ability to build up a complex 3D biofilm contributes to the high resistance of biofilm to ATs [37,43]. Antibiotic resistance would be related to both, adherence of cells to surfaces and bacterial aggregation. However, the adherence would not be a sufficient condition for resistance and bacterial aggregation seems to be needed in order to guarantee this effect. In agreement with these observations, it has been proposed that high-density planktonic growth as well as aggregates in suspension are able to stimulate the same level of resistance than biofilms [38].

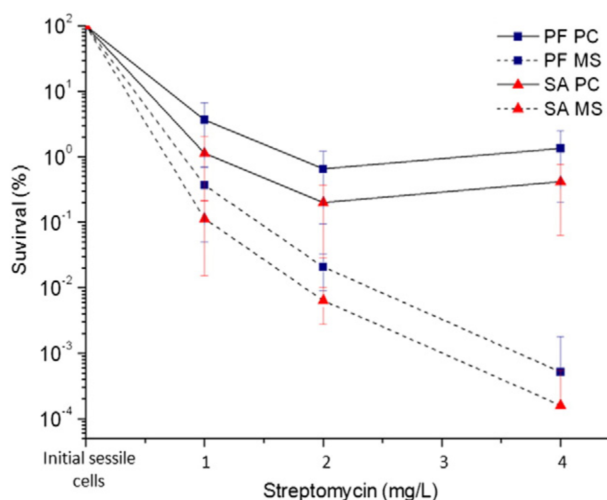

Fig. 4. Effect of streptomycin on viability of *S. aureus* and *P. fluorescens* cells on PC and MS substrates. Results are expressed as mean values SD. Data corresponding to *P. fluorescens* were taken from ref. [26].

Present results enforce the hypothesis that high density of bacteria and a minimum number of bacteria in aggregates are necessary both, to protect themselves initially from the aggressive environment without interrupting their growth and thereafter, when the required threshold value of bacteria is reached, to trigger quorum sensing that induces persisters generation [21,40,42,45]. Bacterial networks built on PC could be considered as a dense growth mode [37] able to survive and develop 3D structures with a density enough to accumulate signaling molecules and induce phenotypic transformations in persisters [20,22]. It should be taken into account that the network is formed by a monolayer of bacteria that is a homogeneous collection of surface-attached cells whose formation is synchronized with cell division [14]. Conversely, cells adhered on MS (even with initial number of cells similar to those attached on PC) experienced a higher killing rate than those attached to PC. As a result they could neither promptly generate 3D densely packed structures nor reach the adequate bacterial density to activate quorum sensing mechanism that induces strong persistence. Thus, isolated bacteria and very small aggregates attached on MS could be easily eradicated.

We believe that there are no previous reports about the antibiotic susceptibility of single adherent cells from wild type *S. aureus* strain in early stages of biofilms formation. On this respect, MS surface is an excellent strategy to study isolated sessile bacteria without the use of mutant strains or mechanical separation methodologies that could affect the results.

This simple approach, based on the use of microstructured surfaces specifically designed to keep sessile cells isolated, is also adequate for studies about single sessile cell, such as proteomic analysis. Thus, the expression of proteins associated to bacterial adhesion and aggregation may change when they attach on microstructured or planar surfaces and may be followed by this technique. Moreover, the engulfment and killing action of phagocytes may be different for isolate cells or aggregates formed on both types of surfaces and it is an interesting topic to be analyzed in the near future.

5. Conclusions

The antimicrobial action of streptomycin and levofloxacin against densely adherent *S. aureus* 2D aggregates and isolated sessile cells was analyzed without employing mutated *S. aureus* strain. The responses of 2D bacterial networks formed on smooth PC and isolated sessile cells and that of small aggregates formed on sub-microstructured MS were compared. Resistant behavior of *S. aureus* was found in case of streptomycin treatments when bacteria were grown on PC but it was not achieved on MS. Levofloxacin was more efficient than streptomycin in killing *S. aureus* biofilm cells on PC surfaces. The antimicrobial activity of both antibiotics was enhanced and bacteria on MS did not show a resistant behavior. On this surface, bacteria attached as single cells or as small assemblages, were more sensitive to the antibiotic action than the larger 2D networks formed on PC that could grow and generate resistant 3D structures. The behavior of nonmotile *S. aureus* treated with streptomycin was similar to that previously observed for motile *P. fluorescens* attached on PC and MS surfaces. In this sense we have demonstrated that in the case of isolated sessile bacteria, cell adhesion on surfaces is not *per se* a sufficient condition to guarantee the resistance to antimicrobial agents as it has been previously suggested [37].

Our results enforce the hypothesis that high dense aggregates with a minimum number of bacteria are necessary firstly, to protect themselves from the aggressive surroundings without interrupting their growth and subsequently, after reaching a threshold value of bacterial number they may be able to trigger quorum sensing in order to induce the generation of persisters. Consequently, a good strategy to avoid implant-related infections and to improve AT efficacy should be focused in preventing the initial bacterial aggregation. The use of surfaces with microstructural patterns, has proved to be a suitable strategy that fulfills two purposes: to inhibit cell adhesion and aggregation and enhance antimicrobial efficacy against the early stages of biofilm formation of bacteria (motile and non-motile).

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Appendix A. Supplementary data

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