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ORIGINAL ARTICLE

Antibacterial effect of antibiotic-loaded SBA-15 on biofilm formation by *Staphylococcus aureus* and *Staphylococcus epidermidis*

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Staphylococcus aureus **and** ***Staphylococcus epidermidis*** **are human pathogens involved in implant-related infections. During those diseases, they are able to form biofilms showing resistance to the effect of many different antibiotics. Drug delivery systems allow a local and effective delivery of antibiotics at high concentrations in the infected tissue without causing the cytotoxic effects commonly linked to systemic administration. We report the use of a porous ceramic biomaterial, such as SBA-15 loaded with antibiotics, to deliver them directly to the infected tissue. SBA-15 discs were loaded with Vancomycin, Rifampin and a combination of both, introduced in a suspension of** ***S. aureus*** **15981 and** ***S. epidermidis*** **ATCC 35984 and incubated during 6 and 24 h. A statistically significant decrease in the biofilm density and the number of viable bacteria was detected for all antibiotics at 6 h in both bacteria. Rifampin showed an increase in the biofilm density and the number of viable bacteria at 24 h. No differences were detected between Vancomycin and the combination of antibiotics.** ***S. epidermidis*** **was more sensitive to the effect of the antibiotics than** ***S. aureus*****. Here we have demonstrated that SBA-15 is able to act as an effective drug delivery system not only from a pharmaceutical point of view, but also from a biological one.**
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INTRODUCTION

The use of biomaterials in orthopedic surgery or implants to repair a bone fracture has represented an improvement in the life of millions of patients throughout the world.[1] Prosthesis are made of biomaterials, which can be defined as materials destined to interact with biologics systems in order to assess, to treat, to increase or to replace some body tissue, organ or function.[2]

However, the use of prosthesis is not free from complications, which include an increase in the susceptibility to infection. This takes place when bacteria colonize the surface of the prosthesis and subsequently form a biofilm.[1] The treatment against those bacteria includes periods of antibiotic therapy, in many cases using a parenteral way of administration.[3] Local release could lead to high levels of antibiotic in the infected tissue without the development of systemic toxicity. Nowadays, antibiotic-loaded polymethylmethacrylate is commonly used, but with some limitations, because only certain antibiotics can be employed and their release is not always controlled.[4]

One of the commonly used family of biomaterials used in orthopedic surgery are ceramics. They show interesting characteristics such as absence of toxicity, inflammatory response and immune reaction. Those bioceramics are currently used for bone filling, bone replacement and as part of certain implants.[1,5] In addition to these properties, porous ceramic materials can be used as Drugs Delivery Systems (DDS), because they allow loading different antibiotics that can be subsequently released only in the infection focus.[6] In this sense, silica-based ordered mesoporous materials have recently shown a great potential as drug carriers and in bone regeneration. These matrices present a network of cavities within the silica matrix structure with an ordered distribution of the mesoporous, with high surface areas (*ca.* 1000 $m^2 g^{-1}$), tuneable pore size (2–10 nm) homogeneous pore morphology and high pore volume (*ca.* 1 $cm^3 g^{-1}$). One of the main advantages of using those materials for drug delivery is the drug loading capacity, since mesoporous materials can load a great amount of different molecules in comparison with other drug delivery systems. In addition, the release kinetics can be tuned through an easy organic modification of the surface of the matrices.[7,8]

Staphylococcus aureus and *Staphylococcus epidermidis* are bacteria of the human microbiome that are frequently involved in implant-related infections, where these ones are the commonest isolated genus.[9,10] We have previously reported the use of a ceramic biomaterial (SBA-15) as a carrier of antibiotics,[4] and here we report the actual effect of antibiotic release in biofilm development by the clinically relevant species of staphylococci.

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MATERIAL AND METHODS

SBA-15 ordered mesoporous materials were prepared as previously reported.[11] Briefly, 4 g of Pluronic P123 (a polyethylene oxide–polypropylene oxide block copolymer) were dissolved in 104 ml of water and 20 ml of 37% HCl under magnetic stirring. After dissolution of the surfactant, 9.16 ml of tetraethyl orthosilicate (TEOS) was added to the surfactant solution, which led to a molar composition of TEOS:P123:HCl:H_2O of 1.0:0.017:6.03:145. This solution was magnetically stirred at room temperature for 12 h and subsequently aged at 100 °C in sealed polytetrafluoroethylene containers for 24 h. The obtained mesoporous particles were then filtered, washed with water and dried at 60 °C overnight. After surfactant removal by a thermal process at 550 °C, ordered mesoporous silica-based materials were obtained, as confirmed by small angle X-ray diffraction and N_2 adsorption analysis.[12,13] SBA-15 discs were prepared with 150 mg portions of the material compacted through uniaxial (2 MPa) and isostatic (2 MPa) pressure to obtain disk pieces with a diameter of 6 mm.[4] The discs were loaded with Vancomycin, Rifampin and a combination of both according to the protocol described by Molina-Manso *et al*.[4] For this purpose, SBA-15 discs were incubated with 2 ml of antibiotic solution (333 mg l^{-1} for each antibiotic[4]) in phosphate buffered saline (PBS) during 24 h at 4 °C with constant agitation. Preparation of this solution was described in detail by Molina-Manso *et al*.[4] After this period, loaded discs were preserved at 4 °C.

Microbiology studies have been performed using laboratory strains *S. aureus* 15981[14] and *S. epidermidis* ATCC 35984. Discs were incubated in a bacterial suspension with *S. aureus* 15981 and *S. epidermidis* ATCC 35984. The experiments of staphylococcal biofilm formation were carried out in SBA-15 without antibiotics as a control (SBA-C), SBA-15 doped with Rifampin (SBA-RA), SBA-15 doped with Vancomycin (SBA-VA) and SBA-15 doped with both antibiotics (SBA-RA/VA). All antibiotics were purchased from Sigma-Aldrich (St Louis, MO, USA). The experiments were performed following the protocol developed by Kinnari *et al*.[6] Bacteria were inoculated in Tryptic Soy Broth (TSB, bioMérieux, Marcy L'Etoile, France) and incubated overnight at 37 °C with 5% CO_2. After culture, bacteria were centrifuged for 10 min at 3.500 *g* at 22 °C. Supernatant was then discarded and the pellet was washed three times with sterile PBS. Bacteria were then suspended and diluted in PBS to obtain a 10^8 CFU ml^{-1} concentration.

Biofilm formation was obtained incubating discs in a 0.5 McFarland bacterial suspension of *S. aureus* or *S. epidermidis* for 6 and 24 h at 37 °C in 5% CO_2 atmosphere. Afterwards, discs were introduced in 10 ml of PBS and vortexed for 30 s, sonicated for 2 min and vortexed for additional 30 s. Serial dilutions of the samples in PBS were made and incubated using the drop plate method in plates at 35 ± 2 °C.[15] The experiments were repeated three times per each SBA-15 doped with different antibiotics (Rifampin, Vancomycin and Rifampin plus Vancomycin). To analyze the biofilm formed on the samples, cell enumeration was performed using the mathematical formula of the standard test method approved by ASTM (Designation: E 2196-12).[15]

Minimum inhibitory concentration of Rifampin and Vancomycin was obtained by Epsilon test methodology (bioMérieux, Marcy l'Etoile, France) for both original strains and for bacterial isolates obtained after 6 and 24 h to evaluate the emergence of antibiotic mutants. The test was performed according to the instructions provided by the supplier.

Planktonic cell enumeration

After incubating discs with the bacterial suspension for 24 h for biofilm formation, the solution was collected from the 6 × 4 plate for quantification and susceptibility study of planktonic bacteria. Serial dilutions in PBS were made and then incubated using the drop plate method in plates at 35 ± 2 °C. These experiments were repeated three times.

Statistical analysis

Statistical analysis of the results was performed using the EPI-INFO 3.5.4 software (CDC, Atlanta, USA). Bartlett's test was used for the evaluation of the inequality of population variances, and Kruskal–Wallis test was used for comparison of non-homogeneous variances. Significance level was established at $P<0.05$.

RESULTS

Evaluation of biofilm development on antibiotic-loaded SBA-15

S. aureus biofilms on the disc showed a biofilm density reduction in the SBA-15 doped with antibiotics against the control (SBA-C) non-treated samples ($P<0.05$, Kruskal–Wallis test in treated samples and $P=0.82$ in non-treated samples) (Figure 1).

After 6 h, there was no statistically significant difference on the biofilm density between SBA-RA/VA and SBA-RA or SBA-VA ($P>0.05$, Kruskal–Wallis test). After 24 h of incubation, the sample doped with SBA-RA achieved the similar density that the control sample. However, SBA-VA and SBA-RA/VA showed significant decrease in the results ($P<0.05$, Kruskal–Wallis test).

Comparing each of the antibiotics at 6 and 24 h, SBA-RA samples showed less biofilm density than the other samples at 6 h (Figure 1). Biofilm density of SBA-VA and SBA-RA/VA is less at 24 h than at 6 h. Statistics analysis showed that the most significant differences between the studied times were achieved with SBA-RA.

For *S. epidermidis*, at 6 h, statistical analysis shows that there was a statistically significant decrease ($P<0.05$) for biofilm density (measured as number of viable bacteria released by sonication from the disks) when comparing SBA-RA and SBA-VA against the control sample (Figure 2).

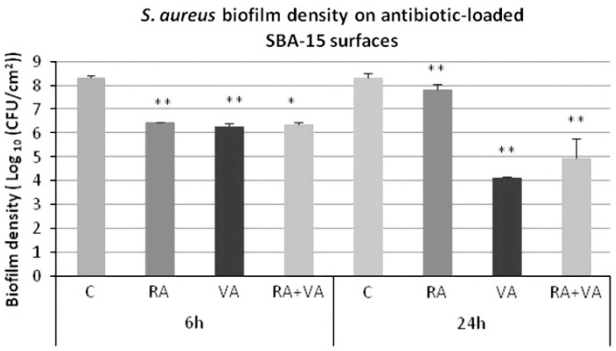

Figure 1 *S. aureus* biofilm density at 6 and 24 h for all the tested materials. $^{**}P<0.05$ when compared against two surfaces; $^{*}P<0.05$ when compared against one surface. C, control SBA-15 without antibiotics; RA, rifampin-loaded SBA-15; RA+VA, rifampin+vancomycin-loaded SBA-15; VA, vancomycin-loaded SBA-15.

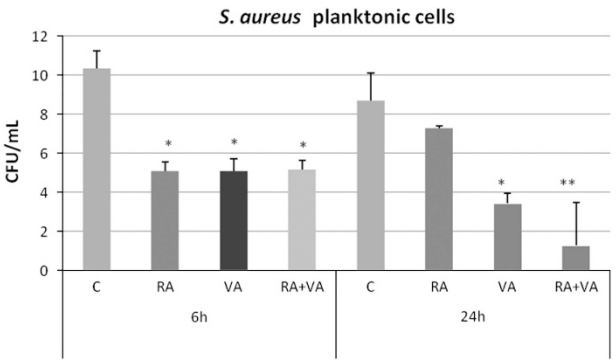

Figure 2 *S. epidermidis* biofilm density at 6 and 24 h. $^{**}P<0.05$ when compared against the control. C, control SBA-15 without antibiotics; RA, rifampin-loaded SBA-15; RA+VA, rifampin+vancomycin-loaded SBA-15; VA, vancomycin-loaded SBA-15.

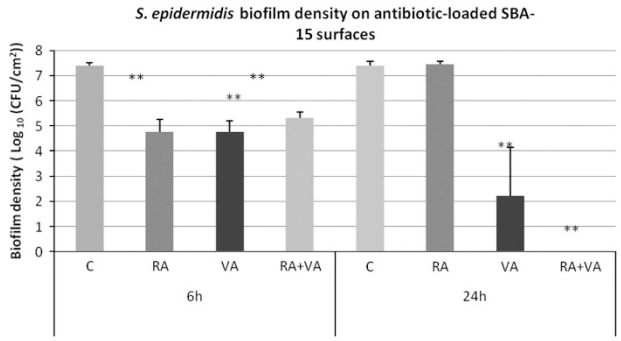

Figure 3 Number of *S. aureus* viable cells at 6 and 24 h. $^{**}P<0.05$ when exists a difference against the control and other sample; $^{*}P<0.05$ when exists a difference against the control. C, control SBA-15 without antibiotics; RA, rifampin-loaded SBA-15; RA+VA, rifampin+vancomycin-loaded SBA-15; VA, vancomycin-loaded SBA-15.

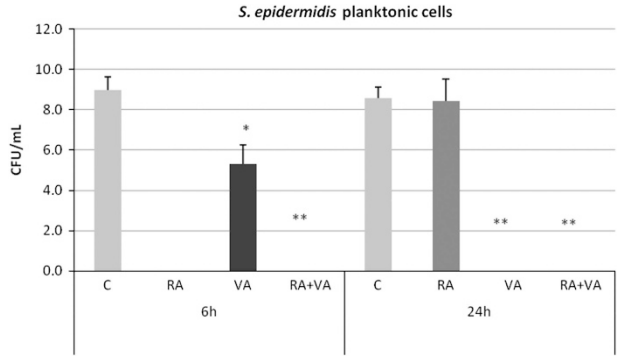

Figure 4 Number of *S. epidermidis* viable cells at 6 and 24 h. $^{**}P<0.05$ when exists a difference against the control and other sample; $^{*}P<0.05$ when exists a difference against the control. C, control SBA-15 without antibiotics; RA, rifampin-loaded SBA-15; RA+VA, rifampin+vancomycin-loaded SBA-15; VA, vancomycin-loaded SBA-15.

Evaluation of planktonic cells

From a global point of view, statistics results showed a significant reduction of the number of *S. aureus* viable cells between control and 6 and 24 h ($P<0.05$, Kruskal–Wallis test, Figure 3).

At 6 h, treated-samples achieved a statistically significant decrease in the number of viable organisms in comparison to the control sample ($P<0.05$), but no such statistically significant differences were found when compared all treated-samples. At 24 h, the number of cells in Vancomycin sample is almost similar to control sample with a *P*-value >0.05. Vancomycin and combination samples continued to show *P*-value <0.05. For *S. epidermidis*, no growth was observed in Rifampin and combination samples at 6 h. Vancomycin sample showed a significant reduction when compared with the control. At 24 h, no growth was observed only in combination sample. However, Rifampin sample showed growth, reaching a similar level than control sample (Figure 4)

E-test susceptibility testing

Minimal inhibitory concentration of strains before incubation and after 6-h incubation period was identical for both *S. aureus* (0.003 mg l^{-1} for Rifampin and 1 mg l^{-1} for Vancomycin) and *S. epidermidis* (0.047 mg l^{-1} for Rifampin and 1.5 mg l^{-1} for Vancomycin). However, after 24-h incubation period with Rifampin, both strains became resistant to this antibiotic (MIC >256 mg l^{-1}). This resistance does not appear with the combination of both antibiotics. No resistance against Vancomycin was detected.

DISCUSSION

The treatment for implant-related infections is a combination of medical and surgical procedures that often includes implant removal.[2] An alternative to this approach could be the use of DDS that allows local release of antibiotics, which leads to a high local concentration without systemic effects. This leads to a greater effect on infection.[16] In addition, these biomaterials can be loaded after their synthesis, avoiding any potential antibiotic degradation during the synthesis of ceramics.[3] The drug loaded could also be chosen depending on the patient's infection or necessity, which opens the gates to personalized medicine.[17] We have previously evaluated the characteristics of SBA-15 as a DDS with Vancomycin, Rifampin and Linezolid in a previous report,[4] where we have also demonstrated that SBA-15 can load these antibiotics, alone and in combination, with a release dynamics that show almost complete release of the antibiotics after 24 h. Moreover, we have shown not only the release of antibiotic molecules, but also the biological activity of the released antibiotics.[4] In addition, we have also tested the capacity of this material to load other molecules that can increase osteoblastic growth and bone regeneration.[18] However, despite these results, it is important to consider some aspects of the study. First, the rapid release of the antibiotics[4] implies that their effect could be useful only in the first hours since implantation, albeit material functionalization that allows sustained antibiotic release could be a potential approach to improve this property.[19] In second place, the amount of bacteria present in the infectious focus is extremely variable. Recent studies showed that bacterial counts in prosthetic joint infections could range between $<10^3$ CFU ml^{-1} and $>10^5$ CFU ml^{-1}, depending from many variables, including the type of implant and previous antibiotic treatments.[20,21] However, the amount of bacteria that are present during surgery is quite lower,[22,23] so this material could be useful to prevent infections in orthopedic patients. The fact that we have detected a statistically significant difference between antibiotic-loaded material and the unloaded one even with the high inoculum used in this experiment support the potential effect for prevention of infection, where low amount of bacteria are probably present.

There are studies that demonstrate the importance of Rifampin on the antibiotic treatment of staphylococcal infections.[24,25] However, disadvantages appear when Rifampin is used in monotherapy because the development of resistance, as we have demonstrated in our study, where the development of a homogeneous bacterial population with high degree of Rifampin resistance was detected between 6 and 24 h. This could be explained by the selection of resistant mutants under selective antibiotic pressure.[26] An alternative option to avoid the emergence of resistant mutants was the combination of Rifampin with other antibiotic.[26] Lucet *et al.*[27] observed that Vancomycin was not enough to avoid the appearance of such resistances, but in this work, when have demonstrated that the association between both antibiotics avoid such selection, although no clear synergistic effect was detected for the bactericidal activity of the combination compared with Vancomycin alone for biofilm-embedded bacteria (Figure 1), but such synergy appear for planktonic *S. aureus* organisms. However, for *S. epidermidis*, synergy was detected for both forms of organisms after 24 h of incubation. These results are

of interest because combined therapy are commonly used in the treatment of some implant-related osteoarticular infections, and the use of SBA-15 loaded with specific antibiotics could improve the treatment of these infections in an individualized way, because antibiotics selected for each specific strain could be selected and then the material used for local release of these ones in the infection focus.

One of the main limitations of our study is the high inoculum used in the experiments, compared with the theoretically low inoculum that could be the cause of implant-related infections. In this sense, a lower inoculum could give different results (especially concerning to the selection of Rifampin-resistant mutants). Another limitation is the selection of the antibiotics. We have selected Vancomycin because most staphylococcal strains are susceptible,[28] and also Rifampin for its good activity against staphylococcal biofilms.[29] However, other combinations could have been tested, like Levofloxacin-Rifampin (a combination recommended for therapy of Meticillin-susceptible *Staphylococcus aureus*) or others. The possibilities of SBA-15 to load different antibiotics made it possible to test these other combinations (including potential antibiofilm agents[30]) in further experiments, as well as the optimal loading capacity of the material needs to be tested with increased concentrations of the selected antibiotics. The different variables that can affect the experiment (like the CO_2 concentration of the atmosphere or the culture medium) need also further experiments to evaluate their importance in the obtained results.

Other questions need also to be solved, such as cytotoxicity of the material. In this aspect, there are reports that suggest its cytocompatibility,[31,32] and even its role as DDS for osteogenic peptides that increases osteointegration of the material.[18] The potential of a combination of these peptides and antibiotics in a functionalized material is a potentially useful property of this material that merits further research in this field.

Finally, *in vivo* experiments will be needed to clarify the actual usefulness of this methodology for the treatment and prevention of osteoarticular infections.

CONCLUSIONS

We have demonstrated the ability of SBA-15 as a DDS with actual biological effect. All selected antibiotics have had significant effect against biofilm density and the number of viable cells in both *S. aureus* and *S. epidermidis* at 6 h. Moreover, we have demonstrated that the selection of Rifampin-resistant mutants lead to a complete substitution of the susceptible population between 6 and 24 h, an effect that can be avoided if Vancomycin is combined with Rifampin. The potential advantage of SBA-15 allowing the selection of antibiotics according to the patient's needs could be used to perform individualized therapy with the selection of the optimal antibiotic combination that avoids the development of resistances and so improves the clinical management of the patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author contributions: AA-C performed the experimental work, the analysis of the data and participates in the manuscript preparation. CP-J participates in the design of the experiment, the analysis of the data and participates in the manuscript preparation. JCD and MM prepared the SBA-15 discs and participates in the manuscript preparation. MV-R and JE designed the experiments; participate in the data analysis and manuscript preparation. All authors approved the final version of the manuscript.

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