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# Biopolymers-Calcium Phosphate Antibacterial Coating Reduces the Pathogenicity of Internalized Bacteria by Mesenchymal Stromal Cells

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## **Abstract**

A multifunctional material system that kills bacteria and drives bone healing is urgently sought to improve bone prosthesis. Herein, the osteoinductive coating made of calcium phosphate/chitosan/hyaluronic acid, named Hybrid, was proposed as an antibacterial substrate for stromal cell adhesion. This Hybrid coating possesses a contact-killing effect reducing by 90% the viability of Gram-positive *Staphylococcus aureus* (*S. aureus*) and Gram-negative *Pseudomonas aeruginosa* (*P. aeruginosa*) strains after 48h of contact. In addition to the production of immunomodulatory mediators, Wharton's jelly (WJ-SCs), dental pulp (DPSCs) and bone marrow (BM-MSCs) derived stromal cells were able to release antibacterial and antibiofilm agents effective against *S. aureus* and *P. aeruginosa* strains, respectively. Studying the effect of the Hybrid coating on the internalization of *S. aureus* by the stromal cells, in acute-mimicking bone infection, highlighted an increase in the bacteria internalization by DPSCs and BM-MSCs when cultured on the Hybrid coating *versus* uncoated glass. Despite the internalization, Hybrid coating showed a beneficial effect by reducing the pathogenicity of the internalized bacteria. The formation of biofilm was reduced by at least 50 % in comparison to internalized bacteria by stromal cells on uncoated glass. This work opens the route for the development of innovative antibacterial coatings by taking into account the internalization of bacteria by stromal cells.

**Keywords:** antibacterial coating, mesenchymal stromal cells, bacteria, *S. aureus* internalization

## Introduction

When prosthesis is implanted in the body, a competition between the host's cells and the bacteria occurs at the implant/bone interface, compromising its osseointegration. Despite strict aseptic procedures, bacterial infections occur and contribute to heavy health and socioeconomic burdens worldwide. Standard treatments for infected bone include the removal of necrotic bone fragments, local and/or systemic administration of antibiotics, and reconstruction of bone defects by bone grafts. These treatments are time consuming, do not always yield satisfactory outcomes, and contribute to bacteria antibiotic resistance emergence.<sup>1,2</sup> The development of biomaterials with intrinsic antibacterial properties was proposed to impede the bacterial growth, to reduce the time of treatment and the total costs. Biomaterial coatings have provided new weapons against bacteria attachment and/or growth. Myriad of surface coatings, based on the anchorage of antibacterial compounds, have been developed, resulting in bacteria membrane disruption and bacteria lysis.<sup>3-8</sup> To our knowledge, up to date, four technologies are available in clinics: (i) silver, (ii) iodine, (iii) gentamicin/poly(D,L-lactide) and (iv) fast resorbable hyaluronic acid/poly(D,L-lactide) hydrogel coatings.<sup>4</sup> Besides antibiotic resistance of bacteria, the genotoxicity and/or cytotoxicity of released ions and the quick resorption of the hydrogel make long-term side effects quite unlikely.

Combining biomaterials with mesenchymal stromal cells (MSCs) has generated significant medical consideration for bone reconstructive and/or regenerative applications. In clinical trials and in terms of regenerative medicine, bone marrow (BM)-MSCs are the gold standard. However, several disadvantages in their use exist including the availability, effectiveness, and invasive and painful procedures required for their isolation.<sup>9,10</sup> MSCs can also be isolated from various tissue types including Wharton's jelly umbilical cord and dental pulp tissues. Wharton's jelly derived stromal cells (WJ-SCs) and dental pulp derived stromal cells (DPSCs) are noteworthy for their ease of accessibility from umbilical cord and healthy teeth (*i.e.* extraction

of non-fully erupted third molars). Regarded as disposable tissues, harvesting cells from umbilical cord or teeth do not raise ethical concerns. WJ-SCs and DPSCs have the capacity for self-renewal and potential for multilineage differentiation,<sup>11-13</sup> constituting ideal candidates to replace BM-MSCs. One of the most intriguing properties of BM-MSCs is their antimicrobial properties responsible of the bacterial clearance in clinical and preclinical models of sepsis,<sup>14</sup> acute respiratory distress syndrome,<sup>15</sup> cystic fibrosis over-infection,<sup>16,17</sup> and secondary infection in patients positively diagnosed with COVID-19.<sup>18</sup> The strong antibacterial effect of BM-MSCs is linked to the release of antimicrobial agents such as cathelicidin LL-37, human  $\beta$ -defensin-2, hepcidin, and lipocalin-2 and also through their immunomodulatory action on the host's immune response.<sup>16,19-21</sup> To our knowledge, no study has been reported so far on the antibacterial properties of either WJ-SCs and DPSCs conditioned media.

Calcium phosphate ceramics have been widely used for bone regenerative medicine due to their ability to induce bone formation.<sup>22-25</sup> We recently elaborated a bioactive and osteoinductive calcium phosphate/chitosan/hyaluronic acid (Hybrid) coating able to boost the early differentiation of stromal cells into osteoblast-*like* lineage. Aside from maintaining the stromal cells paracrine production of osteoprotegerin, Hybrid coating also induces the production of angiogenic growth factors required for bone vascularization, such as the vascular endothelial growth factor.<sup>26-28</sup> Hybrid coating was shown to limit the adhesion of implant-associated *Staphylococcus aureus* (*S. aureus*) without studying the underlying antibacterial mechanism.<sup>26</sup> Prosthesis-related infections are mainly due to contamination by the commensal microbiota of the skin such as *S. aureus*.<sup>29-31</sup> Because of biofilm mode of growth, these bacteria can attach and persist on bone or on implanted prosthesis. Studies dealing with the interaction of *S. aureus* with osteoblasts or stromal cells showed a bacterial adhesion to the cell membranes, followed by their internalization.<sup>29,32-35</sup> *S. aureus* produce microbial surface component recognising adhesive matrix molecules, involved in the bacterial interaction with extracellular matrix

proteins, such as fibronectin<sup>36</sup>. Fibronectin is thought to be used by the bacteria to form a molecular bridge between the bacterial surface proteins and  $\alpha 5\beta 1$  integrin. This latter is detected as early marker on osteoblasts during osteoprogenitor differentiation.<sup>37,38</sup> This could favor the *S. aureus* internalization during the differentiation of stromal cells.

A multifunctional material system that kills bacteria and drives bone regeneration is urgently sought to improve bone prosthesis. To the best of our knowledge, the influence of an antibacterial coating on *S. aureus* internalization by stromal cells and their function was never addressed. Indeed, all the literature dealing with *S. aureus* internalization is focused on the internalization mechanism of different types of cells including osteoblasts and stromal cells cultured on TCPS.<sup>29,32-35</sup> To this end, this work investigates whether Hybrid coating could be used to potentially fight bacterial infection in bone, by killing bacteria and minimising stromal cells/bacteria interaction. We first investigated the antibacterial effect of the Hybrid coating against two bacteria strains, Gram-positive *S. aureus*, responsible for 75% of bone infections,<sup>31</sup> and Gram-negative *Pseudomonas aeruginosa* (*P. aeruginosa*), the most studied bacterium for biofilm formation.<sup>39</sup> Antibacterial and immunomodulatory effects of WJ-SCs and DPSCs (*versus* BM-MSCs) were also tested against both strains. Finally, stromal cells cultured on Hybrid coating were challenged with *S. aureus* to analyze the underlying internalization and the pathogenicity of the internalized bacteria.

## Material and methods

**Materials.** Calcium nitrate ( $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ), diammonium hydrogen phosphate ( $(\text{NH}_4)_2\text{HPO}_4$ ), sodium dihydrogen phosphate hydrate ( $\text{NaH}_2\text{PO}_4$ ), Tris(hydroxymethyl) aminomethane (Tris), calcium chloride hydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ), sodium chloride and chitosan (75–85% deacetylated, low molecular weight) from Sigma and hyaluronic acid (200 kDa) from Lifecore Biomedical were used without further purification. Salt solutions were prepared in ultrapure water (Millipore<sup>®</sup>). For Hybrid coating build-up,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.32 M) and chitosan 0.3 mg/mL were dissolved in NaCl (0.15 M)/HCl (2 mM) buffer, pH 4 whereas  $\text{NaH}_2\text{PO}_4$  (0.19 M) and hyaluronic acid 0.3 mg/mL were prepared in NaCl (0.15 M) buffer pH 10. The sprayed chitosan/hyaluronic molar ratio in monomer is of 2, close to the optimum of build-up.<sup>40</sup> For Inorganic coating build-up, a calcium solution of  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  (0.32 M) and a phosphate solution of  $(\text{NH}_4)_2\text{HPO}_4$  (0.2 M) were prepared in Tris buffer (10 mM Tris, pH 4 and pH 10 respectively). The build-up of Hybrid- and Inorganic coatings was performed on glass coverslip or collagen membrane (Bio-Gide<sup>®</sup>) as described previously.<sup>26,28</sup>

**Physico-chemical characterizations.** Fourier Transform Infrared spectrometry (FTIR) experiments were performed on a Vertex 70 spectrometer (Bruker, Germany) using a DTGS detector. Spectra were recorded in the Attenuated Total Reflection (ATR) mode using single reflexion diamond ATR by averaging 128 interferograms between 600 and 4000  $\text{cm}^{-1}$  at 2  $\text{cm}^{-1}$  resolution, using Blackman-Harris three-term apodization and Bruker OPUS/IR software (version 7.5). Transmission Electron Microscopy (TEM) investigations were performed with a JEOL ARM 200F cold FEG TEM/STEM (point resolution 0.19 nm in TEM mode and 0.078 nm in STEM mode) fitted with a GIF Quantum ER. HR-TEM pictures were performed with a JEOL ARM 200F cold FEG (point resolution 0.19 nm) fitted with a GIF Quantum ER. Finally, scanning electron microscopy-energy dispersive X-ray spectroscopy

investigations were performed with SEM-EDX, JEOL JSM 6010LA. The X-ray spectra were acquired at primary beam energy of 10 keV with an acquisition time of 30 s.

**Coatings' antibacterial properties.** The strains used in the study consisted of *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) wild-type strains from Institut Pasteur Collection (CIP 53.154 and CIP 82.118, respectively). After a preculture of 18 h in nutritive broth, 500  $\mu$ L of minimal medium (MM: 62 mM potassium phosphate buffer, pH 7.0, 7 mM [(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 10  $\mu$ M FeSO<sub>4</sub>] with 0.4 % (w/v) glucose), containing about 10<sup>6</sup> CFU/mL (controlled by enumeration), were deposited on UV-decontaminated (20 min) Hybrid and Inorganic coatings, for 4 h, 24 h or 48 h at 37°C. Both coatings were rinsed with MM, immersed in 2 mL of MM and then sonicated for 5 min. Serial dilutions were further plated on TCS agars plates (Biokar) and colony counts were performed to evaluate adhesive activity.

**Scanning electron microscopy with a field emission gun (FEG-SEM).** Hybrid and Inorganic coatings after 48 h of bacterial incubation were rinsed with MM, before their fixation with 2.5% (w/v) glutaraldehyde (Sigma-Aldrich) at room temperature for 1 h. Samples were then rinsed twice with Phosphate-Buffered Saline (PBS), dehydrated in graded ethanol solutions from 50 to 100% and desiccated in hexamethyldisilazane (Sigma-Aldrich) for 10 min. After air-drying at room temperature, samples were sputtered with a thin gold-palladium film using a JEOL ion sputter JFC 1100 instrument. FEG-SEM investigations were performed with a FEG-SEM (JEOL JSM-7900F, France), and images were acquired from secondary electrons at primary beam energy at 5 kV. Uncoated collagen membrane was used as a control.

**Cell culture.** The studies involving human participants were reviewed and approved by our local Research Institution and were conducted with informed patients in accordance with the usual ethical legal regulations (Article R 1243-57). All procedures were done in accordance with our authorization and registration number DC-2014-2262 given by the National "Cellule



de Bioéthique” of “Agence de Biomédecine”. The patients/participants provided their written informed consent to participate in this study. Human bone marrow (BM) samples were collected by aspiration from femoral necks of patients (aged between 40 and 70 years old) undergoing total hip replacement. Aspirated bone marrow was diluted in Phosphate-Buffered Saline (PBS, v/v) and centrifuged at 300g for 10 min and primary cultures (P0) were then initiated by plating isolated mononuclear cells at  $5 \times 10^5$  cells/cm<sup>2</sup>. Healthy pulp tissues were isolated from the caries-free teeth of patients undergoing extraction of non-fully erupted third molars. Healthy fresh human umbilical cords were harvested after full-term births. Wharton’s jelly derived stromal cells (WJ-SCs) and dental pulp stromal cells (DPSCs) were enzymatically isolated using type B collagenase (1 mg/mL) and trypsin (5 x) mixture. WJ-SCs, DPSCs and BM-mesenchymal stromal cells (BM-MSCs) were all cultured in  $\alpha$ -MEM (Lonza) supplemented with 10 % decompemented fetal bovine serum (FBS), 1 % Penicillin/Streptomycin/Amphotericin B (PSA) and 1 % Glutamax<sup>®</sup> (v/v, Gibco) and maintained in a humidified atmosphere of 5 % CO<sub>2</sub> at 37°C with a medium change every two days. Reaching sub-confluence, BM-MSCs were amplified at density of  $1 \times 10^3$  cell/cm<sup>2</sup> until the second passage, whereas both DPSCs and WJ-SCs were amplified at density of  $3 \times 10^3$  cell/cm<sup>2</sup> until the third passage. WJ-SCs, DPSCs and BM-MSCs were characterized by flow cytometry (BD LSRFortessa flow, BD Biosciences, San Jose, CA, USA) through the expression of CD73, CD90, CD44, CD105, CD34, CD45, and HLA-DR and then used in our experimental procedure at the fourth passage.

**Cells’ antibacterial properties.** Reaching sub-confluence, cells in T75 flask were washed twice with PBS then cultured 72 h in 5 mL of PSA-free  $\alpha$ -MEM medium (basal medium). Cells’ conditioned media (CM) were then collected and conserved at –80°C before use. Diluted overnight cultures of *S. aureus* and *P. aeruginosa* (1/100) were cultured in 100  $\mu$ L of WJ-SCs-CM, DPSCs-CM and BM-MSCs-CM for 24 h at 37°C. The planktonic growth was evaluated

through absorbance measurement at 600 nm, while the formation of biofilm was evaluated by crystal violet staining. Briefly, 96-well plates were gently washed with water and 100  $\mu$ L of 0.2 % crystal violet were deposited per well. After 20 min of incubation, plates were washed with PBS and 100  $\mu$ L of 95 % ethanol were added to each well before absorbance measurement at 595 nm. Basal medium was used as control.

**Cytometric Bead Array (CBA).** WJ-SCs, DPSCs and BM-MSCs were seeded on both coatings at  $24 \times 10^3$  cells/cm<sup>2</sup> in  $\alpha$ -MEM complete medium. After a week of culture, WJ-SCs-CM, DPSCs-CM and BM-MSCs-CM were kept at -20°C before analysis. Secreted levels of interleukin (IL)-8, IL-6 and Monocyte Chemoattractant Protein-1 (MCP-1) were assessed using (BD™ CBA) Human IL-8 Flex Set, Human IL-6 Flex Set, and Human MCP-1 Flex Set (BD Biosciences), respectively, according to the manufacturer's instructions. Results were analysed using LSRFortessa flow cytometer. Results were normalized to CMs of stromal cells cultured on uncoated substrate.

**Cells' infection.** WJ-SCs, DPSCs and BM-MSCs were seeded on build-up coatings (and on uncoated substrate as control) at  $24 \times 10^3$  cells/cm<sup>2</sup> in  $\alpha$ -MEM complete medium. After a week of culture, cells were washed with PBS and cultured, overnight, with  $\alpha$ -MEM PSA-free medium. Cells were then challenged for 3 h with *S. aureus* to obtain a multiplicity of infection (MOI) 30:1.<sup>33</sup> After 3 h of interaction, cells were washed with PBS and incubated for 1 h with basal medium supplemented by 100  $\mu$ g/mL of gentamicin (Fisher scientific). Cells were then lysed with 0.1 % Triton X-100 for 5 min and cell lysates were plated on TCS agars to evaluate the rate of viable intracellular *S. aureus*.

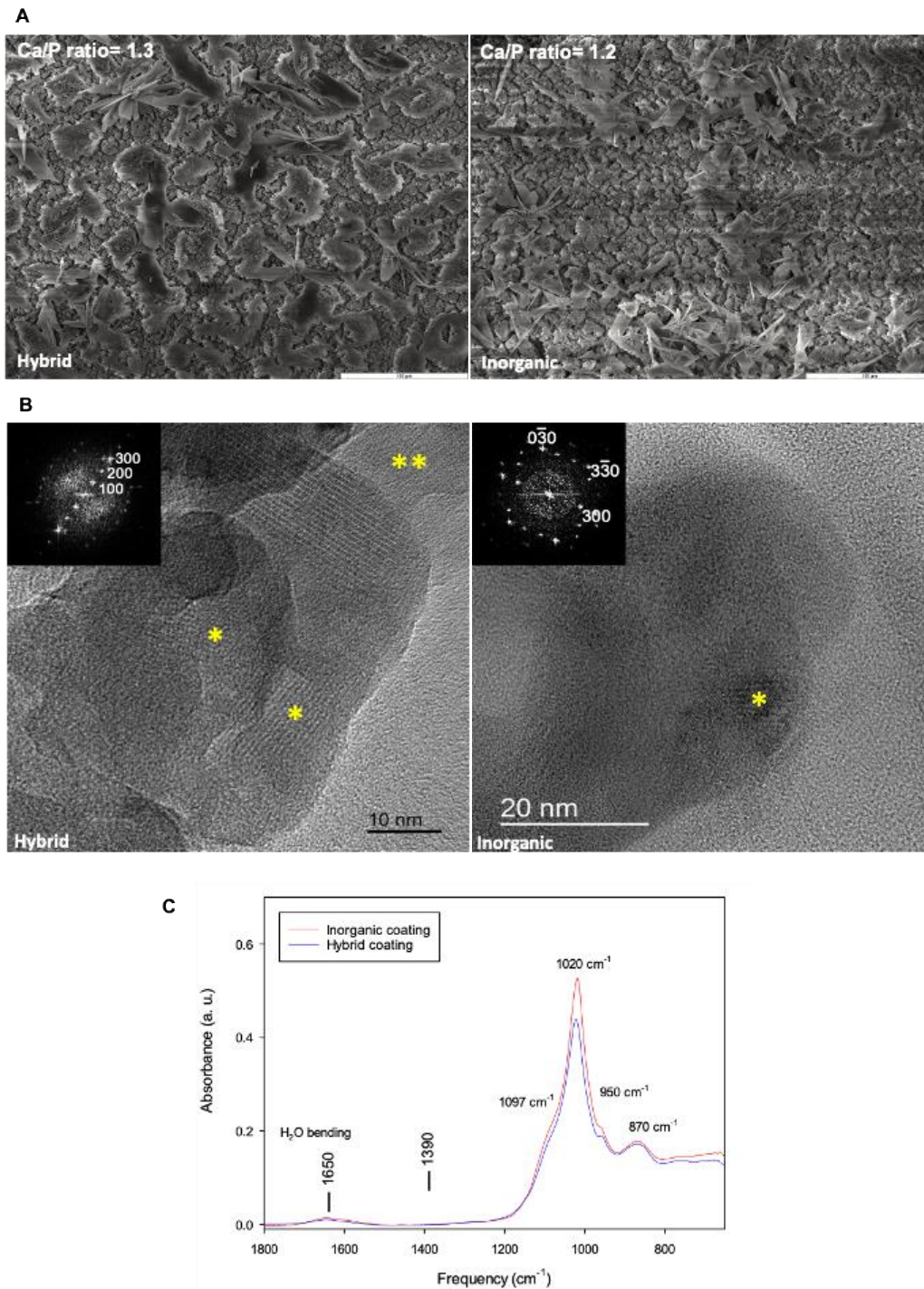
**Biofilm formation by internalized bacteria.** One colony of internalized bacteria (isolated on agar plate after 3 h of infection) was added in 500  $\mu$ L of nutritive broth (Bio-Rad) in 48-well plate for 24 h at 37°C. Plates were then gently washed and biofilm formation was evaluated as

previously described. All results were normalized to formed biofilm by internalized bacteria by cells cultured on uncoated substrate (bacteria control).

**Statistical analysis.** All statistical analyses were performed using GraphPad Prism 6 software. All cell experiments were performed with four independent donors in duplicate. For each bacteria type, at least three independent enumerations were carried out for each surface adhesion experiment and all type of surface was tested in triplicate. Biofilm experiments were performed in triplicate. All results were represented as histograms (mean  $\pm$  SEM), statistical analysis were performed using Mann & Whitney test. For each test, a value of  $p < 0.05$  was accepted as statistically significant  $p$  (rejection level of the null-hypothesis of equal means).

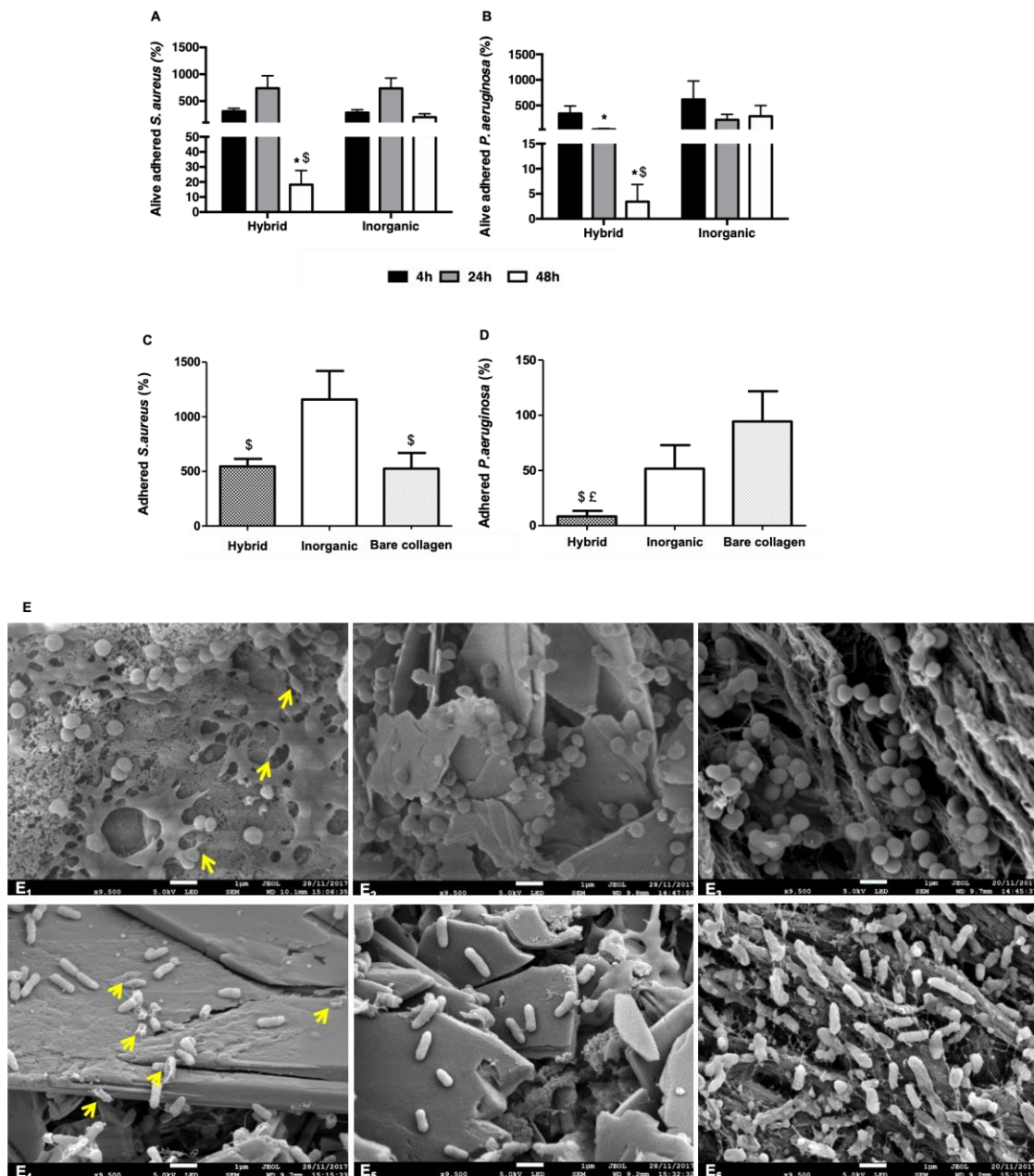
## Results and discussion

**Antibacterial properties of the Hybrid coating.** The Hybrid coating possesses excellent bioactivity and capability of inducing an overwhelmingly positive response of stromal cells and monocytes in favor of bone regeneration.<sup>26-28,41</sup> Obtained by a versatile simultaneous spraying of interacting species, this coating of about 1  $\mu\text{m}$  in thickness is composed of amorphous calcium phosphate and carbonated poorly crystalline hydroxyapatite, with a Ca/P ratio close to 1.30, wrapped within chitosan/hyaluronic acid polysaccharide complex (Fig. 1A and B).<sup>26</sup> Herein, the antibacterial properties of the Hybrid coating was tested against *S. aureus* and *P. aeruginosa*. All the results were compared to the Inorganic coating made of amorphous calcium phosphate and poorly crystalline hydroxyapatite, with a Ca/P ratio close to 1.20.<sup>42</sup> The complete physico-chemical characterization of Hybrid and Inorganic coatings are provided (Fig. 1 and Table SI-1).<sup>26,42</sup> Chitosan/hyaluronic coating showed an unsuccessful build-up on the glass coverslip and was discarded as a control.



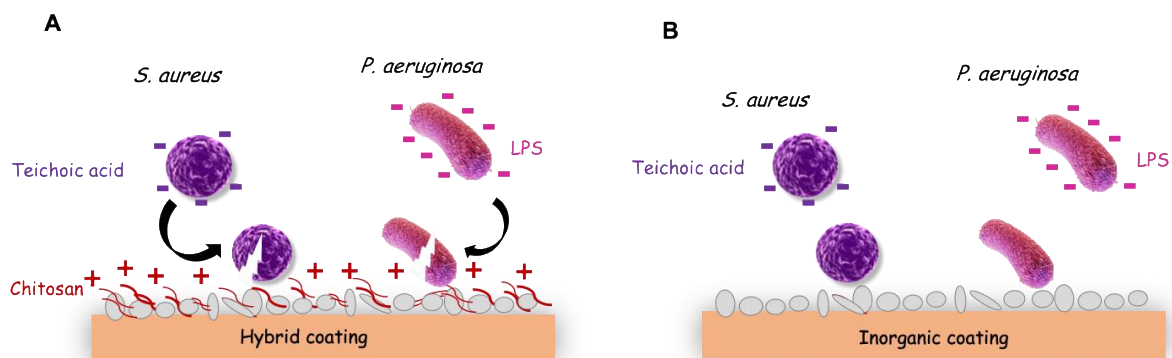
**Figure 1: Morphological and physico-chemical characterizations of the Hybrid and Inorganic coatings.** A: Top views of representative SEM images and Ca/P ratios evaluated by EDX (scale bars indicate  $100\ \mu\text{m}$ ). B: HR-TEM images of the crystal-like structure (inserts represent the extracted FFT pattern and the assigned plans, scale bars indicate 10 and 20 nm, respectively). \* and \*\* indicate mineral crystal and amorphous film, respectively. C: ATR-FTIR spectra of Inorganic and Hybrid coatings. The peaks at 1097, 1020, 950 and  $870\ \text{cm}^{-1}$  are attributed to hydroxyapatite phase.

The percentage of alive *S. aureus* and *P. aeruginosa* on both coatings was monitored after 4, 24 and 48 h of incubation. In all cases, adhered bacteria were detected after 4 h of contact. 24 h later, *P. aeruginosa* were more sensitive to the Hybrid coating in comparison to *S. aureus*, as a sharp decrease in % of alive bacteria was observed (Fig. 2A and 2B). After 48 h of contact, the % of alive *S. aureus* and *P. aeruginosa* were significantly reduced on the Hybrid coating in comparison to the Inorganic one (> 90 % of reduction,  $p < 0.006$ ). When the coatings were built on clinical grade collagen membranes,<sup>28</sup> a significant reduction in *S. aureus* (53 %) and *P. aeruginosa* (87 %) alive bacteria was obtained after 48 h of contact on the Hybrid coating *versus* the Inorganic one (Fig. 2C and 2D). These results suggest that chitosan/hyaluronic acid polysaccharide complexes provide antibacterial properties to the Hybrid coating through a contact killing process.<sup>43,8</sup>



**Figure 2: Antibacterial properties of the Hybrid and Inorganic coating.** (A) Percentage of alive adhered *S. aureus* and (B) *P. aeruginosa* on the Hybrid- and Inorganic coatings over the time (n=4, \* versus previous time; \$ versus inorganic,  $p < 0.05$ ; Mann & Whitney test). (C) Percentage of alive adhered *S. aureus* and (D) *P. aeruginosa* on respectively the Hybrid-, Inorganic coated and bare collagen membranes after 48 h of incubation (n=4, \$ versus inorganic; £ versus bare collagen; Mann & Whitney test). (E) Bacterial adhesion and morphological changes imaged by FEG-SEM, after 48 h of contact of *S. aureus* with the Hybrid- (E<sub>1</sub>), Inorganic coated (E<sub>2</sub>) and bare (E<sub>3</sub>) collagen membranes and of *P. aeruginosa* with the Hybrid- (E<sub>4</sub>), Inorganic coated (E<sub>5</sub>) and bare (E<sub>6</sub>) collagen membranes. Arrows showed *S. aureus* elongation and *P. aeruginosa* cytoplasm contraction on Hybrid coating (Scale bars = 1  $\mu$ m).

Evaluation of bacterial adhesion and morphological changes showed the rupture of the bacterial wall on the Hybrid-coated-collagen membrane. On the uncoated collagen membrane and the Inorganic-coated collagen membrane, bacteria organised themselves in grape-like clusters and kept their structural integrity. *S. aureus* and *P. aeruginosa* morphologies were altered in different way (Fig. 2E). The Hybrid coating induced in *S. aureus* strain an elongation and a disaggregation of grape-like clusters and in *P. aeruginosa* strain a cytoplasm contraction with an increase in wall roughness (Fig. 2E, yellow arrows). The contact-killing modes begin with interactions at the bacteria surface compromising their wall integrity.<sup>43</sup> Hyaluronic acid has antiadhesive and antibiofilm activities<sup>44,45</sup> while chitosan is known to interact with bacterial, causing bacteria wall alterations and death.<sup>46</sup> Polyanionic teichoic acid, ensuring *S. aureus* membrane stability, provides a molecular linkage for the polycationic chitosan, disturbing the cell membrane functions (Scheme 1).<sup>47,48</sup>



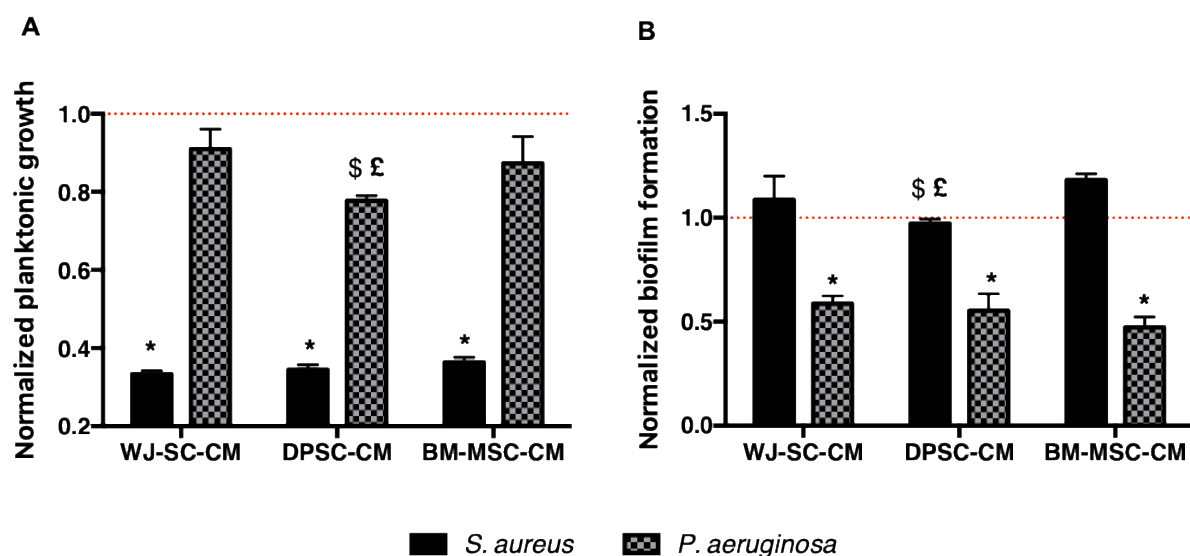
**Scheme 1:** Schematic representation of (A) the contact-killing effect of the Hybrid coating due to electrostatic interactions between negatively charged bacteria cell membrane and chitosan and (B) the no contact-killing effect of the Inorganic coating.



*P. aeruginosa* membrane stability, ensured by interactions between lipopolysaccharide anionic groups (phosphate, carboxyl) and  $Mg^{2+}$  and  $Ca^{2+}$  divalent cations, could be compromised by chitosan, disturbing the bacterial wall or influencing the activity of degradative enzymes. The sensitivity of *P. aeruginosa* compared to *S. aureus* to the Hybrid coating could be explained by the wall architecture, as the lipopolysaccharides density is more important in *P. aeruginosa* wall than teichoic acid density in *S. aureus* one.

**Antibacterial properties of stromal cells.** Several reports showed that BM-MSCs conditioned media (CM) have an antibacterial effect on Gram-positive and Gram-negative strains.<sup>16,20,21,49</sup> WJ-SCs and DPSCs are proposed as an alternative to BM-MSCs in bone reconstructive and regenerative medicine, but no study has been reported so far on the antibacterial effect of either WJ-SCs-CM and DPSCs-CM. Herein, the antibacterial properties of WJ-SCs-CM and DPSCs-CM, harvested after 72 h of culture on uncoated substrate, were investigated against *S. aureus* and *P. aeruginosa* strains. BM-MSCs-CM and cell free medium were used as positive and negative controls, respectively. After 24 h of incubation, *S. aureus* planktonic growth was significantly slowed down in the presence of the three stromal cell-CM *versus* the negative control. *P. aeruginosa* showed a higher susceptibility regarding DPSCs-CM, with about 20 % of reduction in its planktonic growth *versus* the negative control while WJ-SCs-CM and BM-MSCs-CM showed an insignificant decrease (< 10 % of reduction *versus* negative control) (Fig. 3A). Previous report demonstrated a modest antibacterial activity of BM-MSCs-CM against Gram-negative bacteria, which could be, however, enhanced with bacterial challenging.<sup>20</sup> Efficacy of CM in reducing the biofilm formation by *S. aureus* and *P. aeruginosa* strains, on tissue plastic culture substrate (TCPS), was also investigated. After 24 h of incubation, the biofilm formation by *P. aeruginosa* was drastically hindered by the three stromal cell-CM. The biofilm formation by *S. aureus* was increased in the presence of WJ-SCs-

CM and BM-MSCs-CM, while no effect in the case of DPSCs-CM was noticed (Fig. 3B). WJ-SCs and BM-MSCs released factors are thought to provide a source of stress that could reinforce *S. aureus* biofilm formation. Similar antibacterial and antibiofilm activities of the three stromal cell-CM were obtained when cultured on the Hybrid and Inorganic coatings *versus* uncoated substrate (Fig. SI-1). To sum up, whatever the substrate of culture, WJ-SCs and DPSCs have similar release-killing properties as BM-MSCs by secreting soluble factors exhibiting antibacterial and/or anti-biofilm effects. DPSCs seem to be more advantageous as the resulting released factors reduced both *S. aureus* and *P. aeruginosa* planktonic growth and biofilm formation. These results need to be comforted by further investigations through a deep analysis of CM components by peptidomic analysis but this is out of scope of the present study.



**Figure 3:** Antibacterial and antibiofilm effects of conditioned media (CM) from stromal cells culture on uncoated substrate. (A) Planktonic growth of *S. aureus* and *P. aeruginosa* strains in the presence of CM. (B) Biofilm formation of *S. aureus* and *P. aeruginosa* strains in the presence of CM. The results, normalized to cell free culture medium (negative control), indicated by red dashed lines (n=4, \* *versus* negative control, \$ *versus* WJ-SCs-CM, £ *versus* BM-MSCs-CM,  $p < 0.05$  ; Mann & Whitney test).

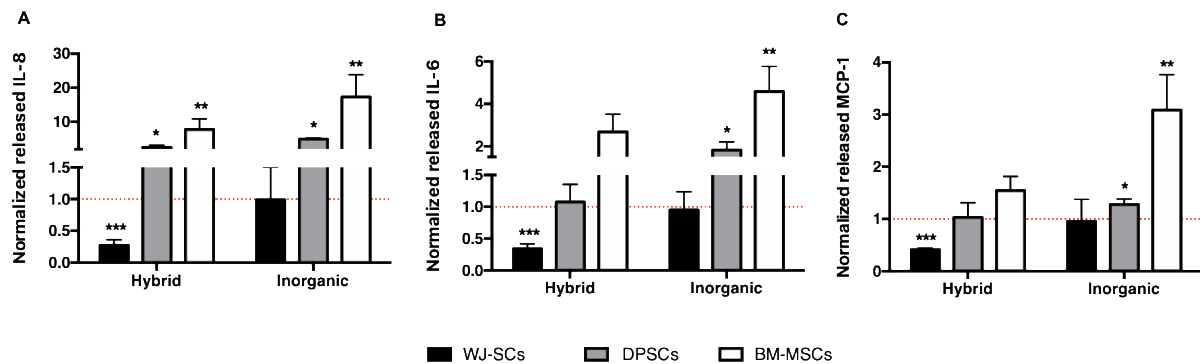
**Immunomodulatory properties of stromal cells.** In the classic acute inflammatory episode, neutrophils are first recruited to the inflammatory site, followed by the infiltration of monocytes and lymphocytes, which replace neutrophils and orchestrate tissue repair. IL-6 and IL-8 are required for a normal neutrophil generation and function.<sup>50</sup> In regard to monocytes, IL-6 and MCP-1 play key roles in regulating monocyte/macrophage phenotypes and activities, favouring anti-inflammatory phenotypes associated with tissue healing. MSCs oppose bacterial infection through the secretion of specific cytokines with improved qualities.<sup>19</sup> We investigated the paracrine activities of WJ-SCs, DPSCs and BM-MSCs cultured on the Hybrid coating. Cells cultured on Inorganic coating and uncoated substrate were used as control. After one week of culture, stromal cell-CM were analysed by CBA. As WJ-SCs showed a higher basal secretion of IL-6, IL-8 and MCP-1 (~ 50 ng/mL) in comparison with DPSCs (~ 0.1 ng/mL) and BM-MSCs (~ 5 ng/mL) (Table 1), results presented in the Fig. 4 were normalized to stromal cell-CM cultured on uncoated substrate.

**Table 1:** Paracrine activities of stromal cells. Released IL-8 (A), IL-6 (B) and MCP-1 (C) measured by CBA.

	IL-8 (ng/mL)		IL-6 (ng/mL)		MCP-1 (ng/mL)	
	Hybrid	Inorganic	Hybrid	Inorganic	Hybrid	Inorganic
WJ-SCs	29.14 ± 2.2	37.16 ± 12.8	14.53 ± 3.1	18.36 ± 3.3	80.67 ± 2.9	115.91 ± 66.2
DPSCs	1.21 ± 0.3	1.96 ± 0.4	0.16 ± 0.07	0.19 ± 0.08	2.34 ± 0.6	2.17 ± 0.4
BM-MSCs	4 ± 1.7	6.09 ± 2.0	8.86 ± 2.9	20.03 ± 2.5	4.23 ± 1.5	5.53 ± 2.1

Interestingly, stromal cells behaved differently in contact with the two tested coatings (Fig. 4). The Hybrid coating reduced drastically the production of IL-8, IL-6 and MCP-1 (< 0.5-fold *versus* uncoated substrate,  $p < 0.0001$ ), while the Inorganic coating did not show any effect. DPSCs cultured on the Hybrid coating increased the IL-8 production (> 2.5-fold *versus* uncoated substrate,  $p < 0.05$ ) with no effect on the two other cytokines. In contrast, DPSCs cultured on the Inorganic coating, exhibited an increase in IL-6, IL-8 and MCP-1 production (> 5-, 2- and 1.2-fold *versus* uncoated substrate, respectively,  $p < 0.03$ ). Finally, BM-MSCs cultured on the Hybrid coating increased the IL-8 production (> 10-fold *versus* uncoated

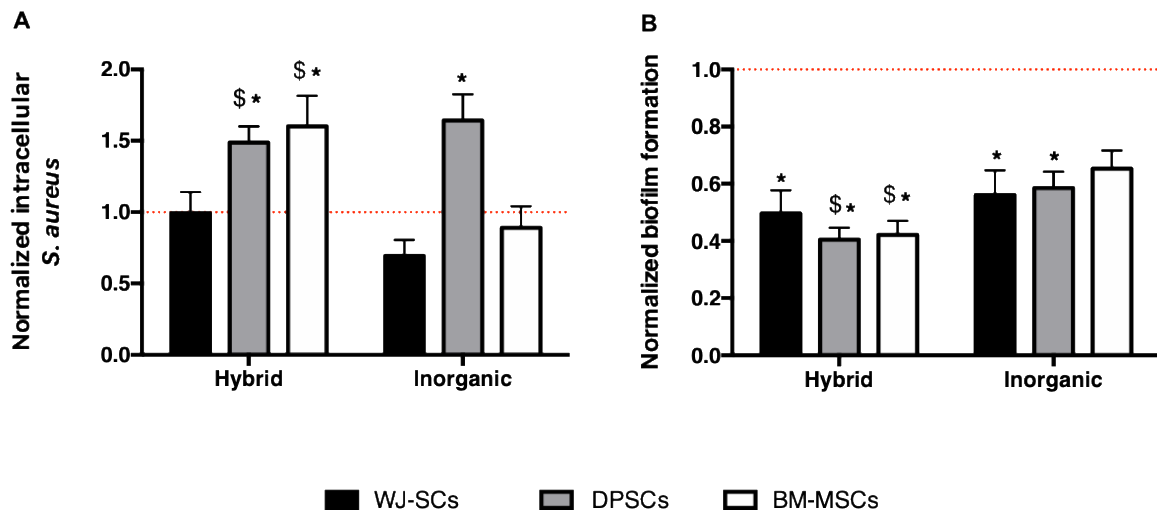
substrate,  $p < 0.01$ ), with an insignificant increase in IL-6 and MCP-1 ones ( $> 4$ - and  $2$ -fold, *versus* uncoated substrate respectively,  $p = 0.09$ ). As DPSCs, BM-MSCs seemed more sensitive to the Inorganic coating as an increase in IL-8, IL-6 and MCP-1 production was noticed ( $> 17$ -,  $5$ - and  $4$ -fold *versus* uncoated substrate,  $p < 0.01$ ). The studied mediators have an important role in controlling the inflammatory response and tissue repair, but at high level, IL-6, IL-8 and MCP-1 could be involved in osteoclast activation, bone destruction and ineffective regeneration.<sup>51</sup>



**Figure 4:** Paracrine activities of stromal cells cultured on the Hybrid and Inorganic coatings. CBA analysis of the released (A) IL-8, (B) IL-6 and (C) MCP-1 in stromal cell-CM. Results were normalized to cytokine level of stromal cells-CM cultured on uncoated substrate, indicated by red dashed lines ( $n=4$ , \* *versus* uncoated substrate  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  ; Mann & Whitney test).

Considering the high level of secreted mediators by WJ-SCs (Table 1), our results suggested that DPSCs, close to BM-MSCs, could be beneficial to harness the acute inflammation. However, the Hybrid coating seems to be beneficial for WJ-SCs as a carrier to regulate the inflammatory process.

***S. aureus* internalization by the stromal cells.** Although the application of bone biomaterials is becoming common, their long-term durability is not guaranteed, and infection remains one of the main reasons for early failure in orthopaedics and trauma.<sup>4</sup> *S. aureus* possesses numerous virulence factors, which play a role in the development and progression of osteomyelitis, through promoting invasion of bacteria, protection of bacteria from host defense mechanisms, and biofilm formation. Herein, we sought to investigate if the antibacterial features of the osteoinductive Hybrid coating provide a protective support for stromal cells, preventing *S. aureus* internalization. WJ-SCs, DPSCs and BM-MSCs cultured on the Hybrid coating were challenged for 3 h with *S. aureus* (acute infective model<sup>33</sup>). Inorganic coating was used as control. The rate of viable *S. aureus* was determined by colony forming unit (CFU) count following gentamicin-protective assay.<sup>33</sup> The bacteria were able to invade the studied stromal cells cultured on uncoated substrate, with a rate of intracellular *S. aureus* of about 1.2 %, 1 % and 0.6 % for WJ-SCs, DPSCs and BM-MSCs, respectively, without any statistical difference between cells (Fig. SI-2A). When cultured on the Hybrid coating (*versus* uncoated substrate), DPSCs and BM-MSCs presented a higher uptake of bacteria, while no effect was observed for WJ-SCs. In comparison to the Hybrid coating, the intracellular rate of *S. aureus* was increased for DPSCs cultured on the Inorganic coating and decreased for WJ-SCs and BM-MSCs (< 1.22- and 1.68-fold *versus* the Hybrid coating,  $p=0.4$  and  $p=0.01$  respectively) (Fig. 5A). To sum up, the osteoinductive and antibacterial Hybrid coating might encourage the *S. aureus* entrance in DPSCs and BM-MSCs but not in WJ-SCs.



**Figure 5: Intracellular rate and biofilm formation of *S. aureus*.** (A) Intracellular rate of *S. aureus* into stromal cells cultured on the Hybrid and Inorganic coatings. Results were normalized to the intracellular rate of bacteria into stromal cells cultured on uncoated substrate, indicated by the red dashed line. (B) Biofilm formation on TCPS by internalized bacteria. Results were normalized to the biofilm formation by internalized bacteria by stromal cells cultured on uncoated substrate, indicated by the red dashed line (n=4, \* versus uncoated substrate, \$ versus Inorganic;  $p < 0.01$ ; Mann & Whitney test).

**Biofilm formation by internalized *S. aureus*.** Biofilm formation is a critical virulence factor responsible for treatment failure and chronicity in orthopaedic device-related infections caused by *S. aureus*.<sup>31</sup> In our laboratory, we observed the occurrence of cell lysis after infection, causing a release of internalized bacteria.<sup>33</sup> Therefore, we evaluated the capabilities of internalized *S. aureus* to form biofilm on TCPS. First, no difference in biofilm formation capability was noticed between non-internalized bacteria and internalized bacteria by stromal cells cultured on uncoated substrate (Fig. SI-2B). In comparison to internalized bacteria by stromal cells cultured on uncoated substrate, a significant decrease in the biofilm formation of internalized bacteria was observed reaching at least 2-fold in reduction for the three stromal cells cultured on both coatings (Fig. 5B). There is an effect of the coating on the pathogenicity of internalized bacteria. Cultured on the antibacterial Hybrid coating, DPSCs and BM-MSCs internalized bacteria had significantly reduced pathogenicity in comparison to the Inorganic

coating. Although the antibacterial Hybrid coating do not prevent *S. aureus* entrance and even favour it in certain cases, the internalized *S. aureus* had weaker capabilities to form biofilm in comparison to those internalized by stromal cells on uncoated substrate.

## **Conclusion**

The antibacterial Hybrid coating, based on calcium phosphate and polysaccharides, provides contact-killing properties by disturbing the cell wall integrity of Gram-positive and Gram-negative bacteria. Its combination with stromal cells, able to release antibacterial agents and mediators of the innate immune response, constitutes an excellent strategy for fighting bacteria. Although the antibacterial Hybrid coating does not prevent *S. aureus* invasion into stromal cells and even favours it in certain cases, the internalized *S. aureus* have weaker capabilities to form biofilm in comparison to internalized bacteria by cells on uncoated substrate. Developing antibacterial strategies for bone tissue regenerative medicine by using stromal cells and/or versatile biomaterials should take into account the cell/bacteria interaction and their internalization.

## **Supporting Information Available:**

The following file is available free of charge and provides a complementary Table related to the characterization of the Hybrid and Inorganic coatings and two Figures related to the antibacterial and antibiofilm effects of conditioned media from stromal cells cultured on the Hybrid and Inorganic coatings, and the biofilm formation by internalized bacteria by stromal cells cultured on uncoated substrate *versus* non-internalized ones.

### **Author Contributions:**

M. Dubus, J. Varin, P. Prada, L. Scomazzon and H. Rammal participated in experiments designing and performance. M. Dubus and H. Rammal in substrate coatings and stromal cell culture, J. Varin (cell infection and scanning electron microscopy), P. Prada and L. Scomazzon (CBA experiments and biofilm). H. Alem (Transmission electron microscopy). F. Reffuveille, F. Boulmedais, C. Mauprivez and H. Kerdjoudj participated in study design and manuscript writing. All authors reviewed the results and approved the final version of the manuscript.

**Conflicts of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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