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Original article

Does low hydroxyl group surface density explain less bacterial adhesion on porous alumina?☆

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ABSTRACT

Background: Bacterial adhesion depends on surface materials. Recently it was suggested that ceramic-on-ceramic bearings could be less prone to infection than other bearings. We examined the possibility that porous alumina ceramic could be less susceptible to bacterial adhesion.
Hypothesis: As hydroxyl groups (OH) on material surface are a major factor governing the surface properties (for example: adsorption, first non-specific step of bacterial adhesion), we hypothesized that alumina had lower OH group density than other material. Thus, we asked (i) if bacterial adhesion was lower on alumina than on titanium alloy, stainless steel and polyethylene and (ii) if OH group density was also lower on alumina.
Material and methods: We performed (i) in vitro bacterial cultures on porous alumina, titanium, stainless steel and polyethylene using *Staphylococcus aureus* and *Pseudomonas aeruginosa*, known to adhere to surfaces. Bacterial cultures were done 3 times in duplicate for each material and each strain. Colony Forming Units (CFU) per cm^2 were measured; (ii) Neutral red reagent helped obtaining OH density estimates using spacer arms. UV-visible spectrophotometry method with Neutral red test, reproduced twice for each surface, provided $\mu g/cm^2$ measurements of OH density.
Results: There was significantly less *P. aeruginosa* adherent on porous alumina (2.25×10^4 CFU/cm^2) than on titanium (4.27×10^5 CFU/cm^2, $p = 0.01$), on stainless steel (2.44×10^5 CFU/cm^2, $p = 0.02$) and on polyethylene (7.29×10^5 CFU/cm^2, $p < 0.001$). *S. aureus* was significantly less adherent on porous alumina (3.22×10^5 CFU/cm^2) than on polyethylene (5.23×10^6 CFU/cm^2, $p = 0.01$), but there was no difference with titanium (1.64×10^6 CFU/cm^2, $p = 0.08$) and stainless steel (1.79×10^6 CFU/cm^2, $p = 0.1$). There was significantly lower Neutral red grafted on porous alumina (0.09 $\mu g/cm^2$) than on titanium (8.88 $\mu g/cm^2$, $p < 0.0001$), on stainless steel (39.8 $\mu g/cm^2$, $p = 0.002$) and on polyethylene (4.5 $\mu g/cm^2$, $p < 0.01$). However, no correlation was found between bacterial adherence and OH group density.
Discussion: Bacterial adherence on porous alumina was lower than on other bearings. Although there were less surface OH groups on porous alumina, we failed establishing a statistical correlation between bacterial adherence and OH group density.
Level of evidence: IV, in vitro study.

One major complication of joint prosthesis is infection. Biomaterials colonization requires several steps and bacterial adhesion starts with non-specific steps involving electrostatic and Van der Walls interactions. These first steps depend on the chemical groups present on the material's surface [1,2]. Recently, several works on hip arthroplasty described contradictory results about a reportedly lower infection rate on ceramic-on-ceramic (CoC), in comparison to other bearings [3–6]. Three registry studies analyzed cohorts of >97,000 to >600,000 patients, demonstrating a lower risk of revision for infection with CoC bearing compared to ceramic-on-polyethylene, metal-on-polyethylene or metal-on-metal bearings [3,5,6]. Paradoxically, a French prospective study on the causes for THA failures, found that infection accounted for 16% of revisions in 238 revised THA using ceramic-on-ceramic bearings in comparison to 11% on other bearings [4]. In vitro, a recent study did not find any difference between ceramic and other materials [7]. In

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addition, infection could be greater on porous surface than on dense materials in the acute phase, but lower after tissue invasion by host cells [8]. Besides, it was noticed that porous alumina implants (vertebra cages, gap fillers for opening wedge high tibial osteotomy) presented a strikingly low rate of postoperative infection. Indeed, only one tibial osteotomy wedge has been infected among more than 5,000 implanted devices made of porous alumina [9]. This could be related to the host cellular layer protecting the materials against bacterial colonization. In the acute phase, surface hydroxyl groups (OH) represent a major surface characteristics that could influence bacterial adhesion [10,11]. They play a significant role in the adsorption of organic molecule [10] and also in wetting and surface reactivity features of certain metal oxide [11]. In addition, OH radicals are the only group present on alumina surface due its crystal conformation and surface oxidation [12].

We hypothesized that porous alumina had a lower rate of bacterial adhesion due to lower surface OH radicals density. To explore this hypothesis we asked:

- if bacterial adhesion was lower on alumina than on titanium alloy, stainless steel and polyethylene, and;
- if OH density was lower on porous alumina surface than on other materials.

1. Material and methods

Tested materials were: 316 L stainless steel, anodized titanium alloy (Ti-6Al-4 V), polyethylene (Ultra-high-molecular-weight polyethylene [UHMWPE]) and porous alumina (Al_2O_3). In all, 316 L stainless steel was purchase from Stainless France® (Dannemarie-sur-Crète, France). After being processed, tested pieces were polished, but not passivated. Titanium alloy was supplied by Stainless France® (Dannemarie-sur-Crète, France) and manufactured, anodized (Selenium Médical®, La Rochelle, France) and mirror polished, in the same standards applied to I.Ceram® implantable products. Polyethylene was purchased from Westlake Plastics® (Bondues, France). As for the other products it was machined using the same tools and processes than those used for I.Ceram® implantable prostheses.

The porous alumina is produced by I.Ceram® out of raw alumina powder purchased from Almatis® (Ludwigshafen, Germany). The final product on which tests were performed is sintered, showing more than 99% purity and 60% total porosity of the final volume. As for the other materials, the process used was the same as the one used for clinical use.

We used 1 cm^3 cubes for bacteriological tests and disks (14 mm diameter; 4 mm height) for chemical tests. For dense materials, the contact surface was 6 cm^2 for cubes and 4.84 cm^2 for disks. For porous alumina (Fig. 1), the contact surface was larger and estimated at 97.43 cm^2 for cubes (15.23 times greater than dense material). This latter surface was evaluated based on the inner surface of pores from which the surfaces of interconnections between pores were subtracted. The mean pore radius in our alumina is 450 μm and 141 μm for interconnections. The mean number of interconnections between adjacent pores was 14. The contact surface was calculated as:

$$\text{Number of pores} \times (\text{inner surface pores}) - 14 \times (\text{surfaces of interconnections})$$

This is to explain that a correcting factor of 15.23 was applied to results of porous alumina cubes and disks to allow comparison with smooth biomaterials.

The primary assessment criterion was the rate of bacterial adhesion assessed by the number of Colony Forming Unit per surface unit (CFU/cm^2). Gram-positive bacteria (*S. aureus* CIP 76.25 – also known as ATCC 25923) and Gram-negative bacteria (*P. aeruginosa* CIP 76.110 – also known as ATCC 27853) were purchased from the Institut Pasteur (Paris, France). These strains were cultivated in liquid tryptic soy (pancreatic casein extract 17 g/L, soy flour papaic digest 3 g/L, dextrose 2.5 g/L, NaCl 5 g/L, and K_2HPO_4 2.5 g/L) and incubated at 37 °C overnight under aerobic conditions. They were chosen for their known ability to colonize biomaterials and to form biofilm [13,14].

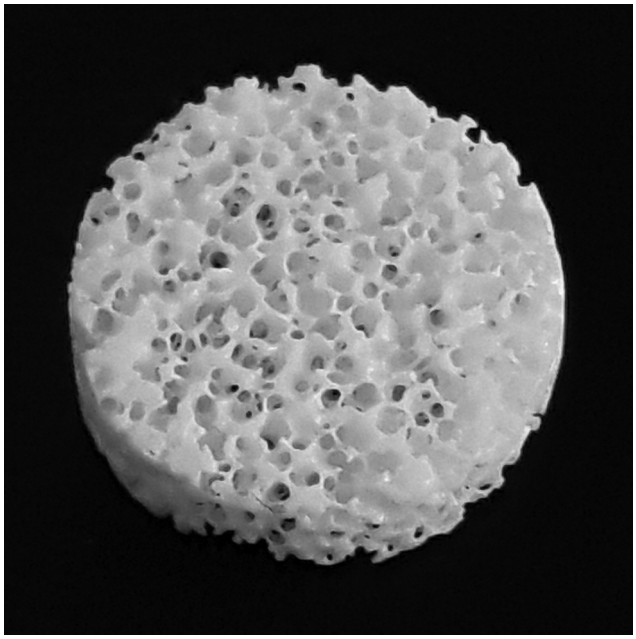


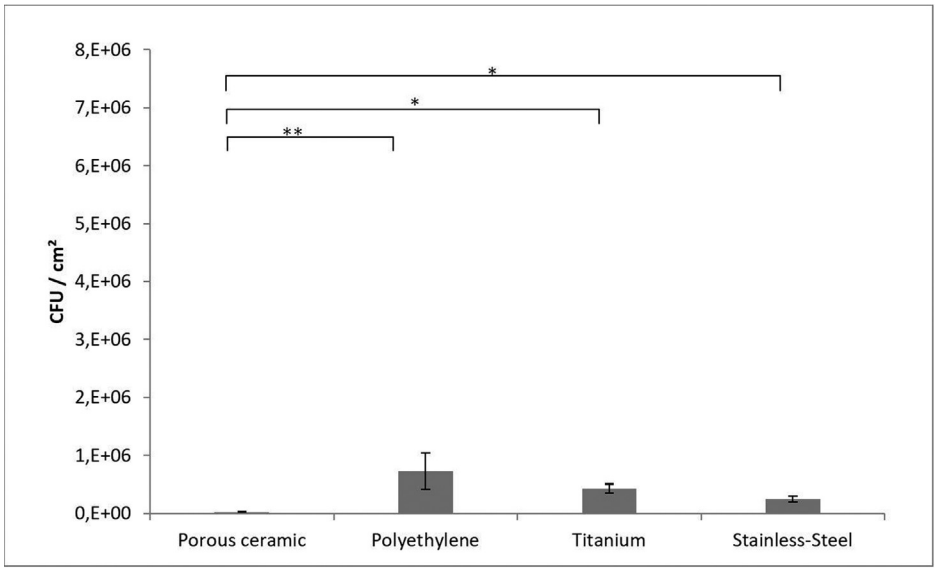
Fig. 1. Porous alumina disk.

Before use, all materials were sterilized by gamma radiation (25 kGy). Sterilized cubes were immersed in 5 mL of bacterial suspension at 5×10^3 CFU/mL concentration for 24 h at 37 °C under aerobic conditions. Then, cubes were rinsed 3 times with physiological water (NaCl 0.9% w/v) in order to eliminate planktonic non-adherent bacteria. Finally, cubes were submitted to 3 cycles of sonication in ultrasonic bath (15 min, 40 kHz) followed by mechanical agitation for 1 min so as to harvest adherent bacteria and seed them on plates.

Each experimentation was repeated three times in duplicate for each type of material and bacterial strain.

Bacterial count was performed after serial dilutions of recovered solution. Each dilution was spread on tryptic soy agar plates using an automatic plater SPIRAL DS® (Interscience®, Saint-Nom la Bretêche, France). After incubation at 37 °C for 24 h, plates were counted to determine CFU/mL and then reported to the surface (CFU/cm^2).

The secondary assessment criterion was the surface OH density assessed using a method which covalently attaches a chromophore specifically onto these groups. Neutral red (ACROS Organics®, pure) was chosen because it can be easily detected by UV-Visible spectrometry (530 nm) (Synergy HTX multi-mode reader, BIOTEK®, USA). It was linked to OH groups using a multi-step reaction. The method is derived from the French Patent Application N° 1761317 filed on 28 November 2017 (under evaluation, not yet published). The amount of grafted Neutral red was determined by indirect quantification, subtracting the amount retrieved in the supernatant of the washing step from the initial amount added for grafting. Whatever the material, each spacer arm bounds to an average of 2 OH groups, so there was a proportional relationship between the amount of Neutral red grafted and the OH density.



*: p<0.05; ** p<0.001

Fig. 2. *Pseudomonas aeruginosa* adhesion to the different tested materials.

Because of the potential adsorption of Neutral red on the surfaces, independently of the presence of the spacer arms, specific adsorption tests were required. Direct addition of Neutral red to disks (same amount used for covalent tethering) in 2-morpholin-4-ylethanesulfonic acid (MES) media at room temperature for 3 hours was performed. Washing and quantification were realized as described above. Each experimentation (with and without spacer arm) was repeated twice for each material. Results are given in $\mu g/cm^2$.

1.1. Statistical analysis

The sample size calculation was based on a 3.5 ± 2.1 folds anticipated bacterial adhesion on tested materials in comparison to porous alumina, with a one-sided alpha = 0.05 and 80% power. Thus, two samples per group were required.

Differences in bacterial adhesion and OH group density to each material were evaluated using Fischer F and Student *t* tests respectively.

Spearman's rank test was used to correlate OH group density and bacterial adherence. Probability values were considered statistically significant if they were inferior to 0.05.

2. Results

There was significantly less *P. aeruginosa* adherent on porous alumina ($2.25 \times 10^4 \pm 6.24 \times 10^3$ CFU/cm^2) than on titanium ($4.27 \times 10^5 \pm 8.35 \times 10^4$ CFU/cm^2, $p = 0.01$), on stainless steel ($2.44 \times 10^5 \pm 5.26 \times 10^4$ CFU/cm^2, $p = 0.02$) and on polyethylene ($7.29 \times 10^5 \pm 3.14 \times 10^5$ CFU/cm^2, $p < 0.001$) (Fig. 2). *S. aureus* was significantly less adherent on porous alumina (3.22×10^5 CFU/cm^2) than on polyethylene (5.23×10^6 CFU/cm^2, $p = 0.01$), but there was no difference with titanium (1.64×10^6 CFU/cm^2, $p = 0.08$) and stainless steel (1.79×10^6 CFU/cm^2, $p = 0.1$) (Fig. 3). Whatever the bacteria, there was no significant difference in adhesion between the 3 other materials. For *P. aeruginosa*: stainless steel vs. polyethylene $p = 0.28$, stainless steel vs. titanium $p = 0.91$ and polyethylene vs. titanium $p = 0.32$. For *S. aureus*: stainless steel vs. polyethylene $p = 0.054$, stainless steel vs. titanium $p = 0.56$ and polyethylene vs. titanium $p = 0.13$.

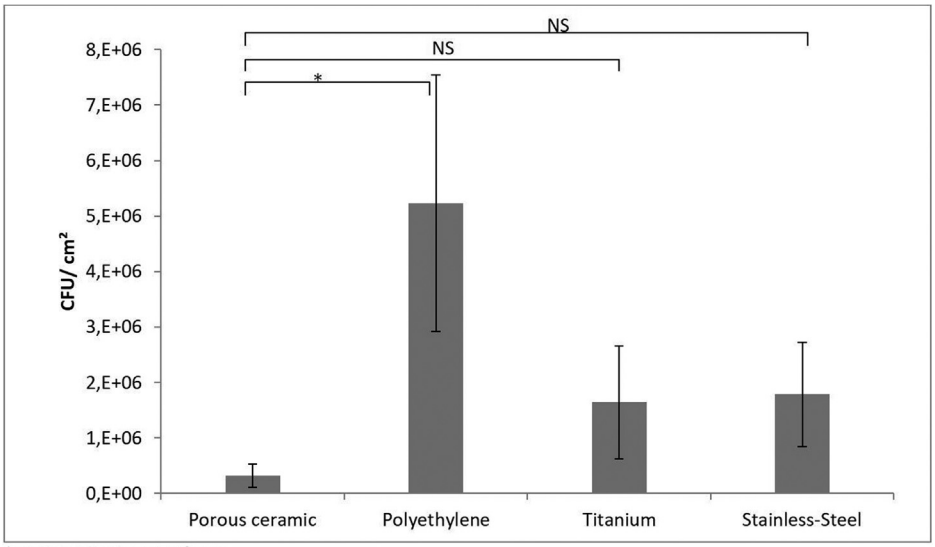
Neutral red was found to be significantly less grafted on porous alumina (0.09 $\mu g/cm^2$) than on titanium (8.88 $\mu g/cm^2$, $p < 0.0001$), on stainless steel (39.8 $\mu g/cm^2$, $p = 0.002$) and on polyethylene (4.5 $\mu g/cm^2$, $p < 0.01$).

In contrast to polyethylene, there was no residual adsorption of Neutral red after ultrasonic cleaning on alumina, titanium or stainless steel (0 versus 6.2 $\mu g/cm^2$), explaining that results concerning the polyethylene had to be discarded from the correlation test. For the remaining bearings, there was no correlation between Neutral red density and bacterial adhesion ($p = 0.75$ with *S. aureus* and $p = 0.9$ with *P. aeruginosa*) (Fig. 4).

3. Discussion

Our in vitro study showed that bacterial adhesion was lower on porous alumina compared to other materials and that OH group density was also inferior on alumina surface:

- *S. aureus* and *P. aeruginosa* were less adherent on porous alumina than on other. Such results were already observed with *S. aureus* showing an even greater difference between alumina and other materials, such as titanium or stainless steel [15]. It might explain a lower risk of infection for CoC bearings compared to other bearings [3,5,6]. On the contrary, a recent in vitro study did not find any adhesion difference between dense alumina and other materials [7]. However, in this publication, sterilization, using steam autoclaving, was repeated between each test using the same devices, which could have modified surface characteristics and bacterial adhesion. Moreover, these authors focused on the initial steps of adhesion (during the first 60 minutes) and ignored long-term colonization with biofilm formation, which might explain their divergent results;
- there was significantly less OH groups on alumina surface using the direct method with spacer arms. Currently, no other method of counting surface radicals is available. X-ray photoelectron spectroscopy (XPS) allows studying materials' surface except on porous structures. Indeed, photo-emitted electrons beam can be lost in porosities causing measurement errors. On the contrary, the technique used in the current study was better adapted to porous surfaces because Neutral red was completely eliminated after washing and ultra-sonication. Thus, only grafted molecules



*: p=0.01; NS: Non-significant

Fig. 3. *Staphylococcus aureus* adhesion to the different tested materials.

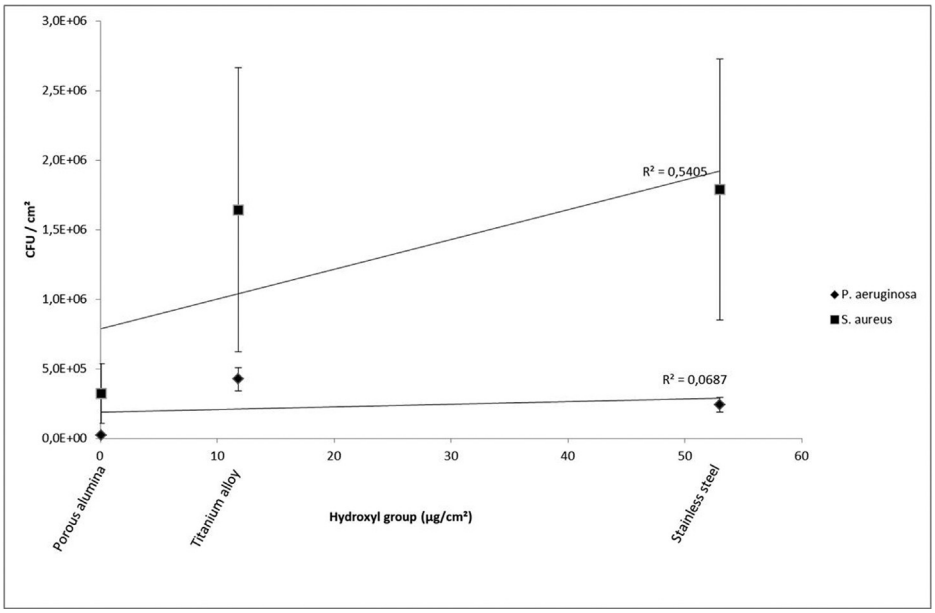


Fig. 4. Plotting of amount of adherent bacteria (*S. aureus* and *P. aeruginosa*) according to hydroxyl group density. Polyethylene results are not plotted because they were not interpretable and used in the spearman correlation test.

were measured, enabling an accurate evaluation of OH group density. This was not the case for polyethylene, because the different chemical groups on its surface vary greatly due to its polymeric composition. Therefore, interactions between close chemical groups might hinder reactions with our spacer arms, leading to false results. In addition, the spacer arm used had a steric hindrance, which does not allow interaction with all groups. The adsorption experimentation revealed the persistence of numerous residual bonds between Neutral red and chemical groups despite sonication and then the biased results obtained with polyethylene.

Even rejecting polyethylene results, there was no clear statistical correlation between OH density and bacterial adherence (Fig. 4). In the literature, it is suggested that OH groups may be involved among many other factors in the first step of adhesion [2,16]. In fact, other characteristics may have an influence such as surface charge, hydrophobicity, microarchitecture and surface roughness [16,17]. In in vivo setting, other factors such as collagen, sialoproteins, fibronectin may act as anchors for bacterial adhesion [18].

Our study presents several limitations. Only OH groups were tested, whereas other chemical substances (polar and apolar groups) are involved in the passive steps of bacterial adhesion [1]. However, OH radicals are the only chemical substances present on alumina surface as a result of its crystalline structure and of the oxidation process. Other proteins may also interact by recognizing adhesive matrix molecules (MSCRAMMs) of the bacterial membrane. However, to date, such conditions cannot be simulated in vitro.

4. Conclusion

We showed in vitro that bacterial adherence on alumina was lower than on the other bearings and that the density of OH groups

was also lower. Although we failed finding a correlation with bacterial adherence, the low density of OH radicals might contribute to the low rate of infections observed with porous alumina devices. Further studies involving proteins are needed to clarify these first results.

Disclosure of interest

E. Denes is an employee of I.Ceram and share stocks.
E. Poli, G. Barrière and G. Lévèque are employees of I.Ceram.
TS. Ouk and V. Sol declare that they have no competing interest.

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Contribution

E. Denes: designed the method, performed immobilization and chemical analyses, manuscript correction.
TS. Ouk: performed bacterial assays, data acquisition, manuscript correction.
G. Barrière: performed bacterial assays, manuscript correction.
G. Lévèque: produced porous alumina, manuscript correction.
V. Sol: performed bacterial assays, manuscript correction.
E. Denes: designed the method, data and statistical analyses, draft manuscript.

References

[1] Arciola CR, Campoccia D, Montanaro L. Implant infections: adhesion, biofilm formation and immune evasion. Nat Rev Microbiol 2018;16:397–409.
[2] Berne C, Ellison CK, Ducret A, Brun YV. Bacterial adhesion at the single-cell level. Nat Rev Microbiol 2018;16:616–27.
[3] Pitto RP, Sedel L. Periprosthetic joint infection in hip arthroplasty: is there an association between infection and bearing surface type? Clin Orthop Relat Res 2016;474:2213–8.
[4] Migaud H, Putman S, Kern G, Isida R, Girard J, Ramdane N, et al. Do the reasons for ceramic-on-ceramic revisions differ from other bearings in total hip arthroplasty? Clin Orthop Relat Res 2016;474:2190–9.
[5] Lenguerrand E, Whitehouse MR, Beswick AD, Kunutsor SK, Burston B, Porter M, et al. Risk factors associated with revision for prosthetic joint infection after hip replacement: a prospective observational cohort study. Lancet Infect Dis 2018;18:1004–14.
[6] Kurtz SM, Lau E, Baykal D, Springer BD. Outcomes of ceramic bearings after primary total hip arthroplasty in the medicare population. J Arthroplasty 2017;32:743–9.
[7] Slullitel PA, Buttaro MA, Greco G, Oñativia JI, Sánchez ML, Mc Loughlin S, et al. No lower bacterial adhesion for ceramics compared to other biomaterials: an in vitro analysis. Rev Chir Orthop Traumatol 2018;104:439–43.
[8] Merritt K, Shafer JW, Brown SA. Implant site infection rates with porous and dense materials. J Biomed Mater Res 1979;13:101–8.
[9] Denes E, Barrière G, Poli E, Lévêque G. Commentary: bioceramics and Scaffolds: a winning combination for tissue engineering. Front Bioeng Biotechnol 2017;5:35–7.
[10] Takeda S, Fukawa M, Hayashi Y, Matsumoto K. Surface OH group governing adsorption properties of metal oxide films. Thin Solid Films 1999;339:220–4.
[11] Takeda S, Fukawa M. Role of surface OH groups in surface chemical properties of metal oxide films. Mater Sci Eng B 2005;119:265–7.
[12] Shirai T, Watanabe H, Fuji M, Takahashi M. Structural properties and surface characteristics on aluminum oxide powders. Annu Rep Adv Ceram Res Cent Nagoya Inst Technol 2009;9:23–31.
[13] Jardeleza C, Foreman A, Baker L, Paramasivan S, Field J, Tan LW, et al. The effects of nitric oxide on Staphylococcus aureus biofilm growth and its implications in chronic rhinosinusitis. Int Forum Allergy Rhinol 2011;1:438–44.
[14] Brown ML, Gauthier JJ. Cell density and growth phase as factors in the resistance of a biofilm of Pseudomonas aeruginosa (ATCC 27853) to iodine. Appl Environ Microbiol 1993;59:2320–2.
[15] Spriano S, Sarath Chandra V, Cochis A, Uberti F, Rimondini L, Bertone E, et al. How do wettability, zeta potential and hydroxylation degree affect the biological response of biomaterials? Mater Sci Eng C 2017;74:542–55.
[16] Ribeiro M, Monteiro FJ, Ferraz MP. Infection of orthopedic implants with emphasis on bacterial adhesion process and techniques used in studying bacterial-material interactions. Biomatter 2012;2:176–94.
[17] Parreira P, Magalhaes A, Gonaçalves IC, Gomes J, Vidal R, Reis CA, et al. Effect of surface chemistry on bacterial adhesion, viability, and morphology. J Biomed Mater Res A 2011;99:344–53.
[18] Hudson MC, Ramp WK, Frankenburg KP. Staphylococcus aureus adhesion to bone matrix and bone-associated biomaterials. FEMS Microbiol Lett 1999;173:279–84.