Influence of surface porosity and pH on bacterial adherence to hydroxyapatite and biphasic calcium phosphate bioceramics

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Hydroxyapatite (HA) and biphasic calcium phosphate (BCP) ceramic materials are widely employed as bone substitutes due to their porous and osteoconductive structure. Their porosity and the lowering of surrounding pH as a result of surgical trauma may, however, predispose these materials to bacterial infections. For this reason, the influence of porosity and pH on the adherence of common Gram-positive bacteria to the surfaces of these materials requires investigation. Mercury intrusion porosimetry measurements revealed that the pore size distribution of both bioceramics had, on a logarithmic scale, a sinusoidal frequency distribution ranging from 50 to 300 nm, with a mean pore diameter of 200 nm. Moreover, total porosity was 20 % for HA and 50 % for BCP. Adherence of *Staphylococcus aureus* and *Staphylococcus epidermidis* was studied at a physiological pH of 7.4 and at a pH simulating bone infection of 6.8. Moreover, the effect of pH on the ζ potential of HA, BCP and of both staphylococci was evaluated. Results showed that when pH decreased from 7.4 to 6.8, the adherence of both staphylococci to HA and BCP surfaces decreased significantly, although at the same time the negative ζ -potential values of the ceramic surfaces and both bacteria diminished. At both pH values, the number of *S. aureus* adhered to the HA surface appeared to be lower than that for BCP. A decrease in pH to 6.8 reduced the adherence of both bacterial species (mean 57 %). This study provides evidence that HA and BCP ceramics do not have pores sufficiently large to allow the internalization of staphylococci. Their anti-adherent properties seemed to improve when pH value decreased, suggesting that HA and BCP bioceramics are not compromised upon orthopaedic use.

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INTRODUCTION

Bioceramics are often implanted in bone-defect areas as bone substitutes due to their osteoconductive behaviour and because they promote bone healing or regeneration or

both by promoting cell growth and proliferation into the porous scaffold. However, the question is whether these materials support not only bone ingrowth but also bacterial adherence. The 'race for the surface' (Gristina, 1987) originally defined for inert orthopaedic implants may be even more important for bioceramics. The most frequently used bioceramics include hydroxyapatite (HA) (Sari *et al.*, 2003; Hench, 1991; Vallet-Regí, 2001) and biphasic calcium

Abbreviations: BCP, biphasic calcium phosphate; CDA, calcium-deficient apatite; HA, hydroxyapatite; β -TCP, β -tricalcium phosphate.

phosphates (BCPs), which consist of a mixture of HA and β -tricalcium phosphate (β -TCP) (Vallet-Regí, 2006; Vallet-Regí & González-Calbet, 2004; Böhner, 2001). BCP shows better performance as a bone substitute than does HA, because the β -TCP component is more soluble and supplies higher amounts of Ca^{2+} and PO_4^{3-} ions than HA does. Furthermore, reports note that the solubility of β -TCP promotes bone ingrowth (Bouler & Gauthier, 1998; LeGeros & LeGeros, 1996). Although their ability to promote bone growth has been well documented, little is known about their susceptibility to implant-related infections.

The first and critical step in the infection process taking place after the surgical procedure is bacterial adherence on biomaterial surfaces (Wadström, 1989). This is a complex process that includes active and passive stages influenced by many factors concerning the bacteria, substratum and surrounding medium. Some physical properties of the substratum, such as surface energy (Gristina, 1987; An & Friedman, 1998), charge (Reynolds & Wong, 1983), hydrophobicity (Hogt *et al.*, 1985; Pringle & Fletcher, 1986), roughness, and configuration (Gristina, 1987; An & Friedman, 1998; Locci *et al.*, 1981), are particularly important. Infection causes some essential changes in the extracellular milieu that can further affect the ability of bacteria to adhere to the substratum. The isoelectric point of the materials at the surface-liquid interface changes upon the decrease in pH following infection, surgery, trauma or aseptic implant loosening (Bessman *et al.*, 1989; Kontinen *et al.*, 2001). On these occasions, the pH of the bone tissue environment often falls below pH 7, whereas in healthy tissues this pH value varies in the range 7.35 to 7.45 (Bessman *et al.*, 1989). This pH decrease is expected to influence the surface charge of biomaterials such as HA and BCP ceramics (Katsikogianni & Missirilis, 2004).

It seems reasonable to assume that these reactive changes in the bone implant environment will influence their susceptibility to implant-related infections. In this work, we hypothesize that the porosity of the bioceramics and the lowering of pH as a result of surgical trauma may predispose these materials to bacterial infections. The present study was therefore performed to determine whether the most common causative agents of orthopaedic implant-related infections, *Staphylococcus aureus* or *Staphylococcus epidermidis*, can penetrate the ceramic pores and adhere particularly avidly to that surface in a slightly acidic pH, simulating conditions to which they may be exposed *in vivo*.

METHODS

Synthesis of HA and BCP materials. Stoichiometric HA and calcium-deficient apatite (CDA) powders were prepared by the crystallization method described elsewhere (Sánchez-Salcedo *et al.*, 2006). Briefly, the powders were prepared by an aqueous precipitation reaction of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (Aldrich) and $(\text{NH}_4)_2\text{HPO}_4$ (Merck), which were simultaneously added dropwise and mixed in a shaker,

giving rise to the following reaction:

where, $x=0$ for HA and 0.73 for CDA. The precipitated powders were formed into 13 mm $\times$ 1 mm discs using uniaxial and isostatic pressure. Afterwards, the HA and CDA discs were treated at 1100 °C and 900 °C for 1 h, respectively, giving rise to the final HA and BCP pieces.

Study of pore size distribution of the HA and BCP materials.

Characterization of the materials was performed by mercury porosimetry recorded in the 5×10^{-3} – 3×10^2 μm range with a Micromeritics AutoPore III 9410 porosimeter (Micromeritics Instruments).

Bacteria. Two biofilm-producing strains, *S. aureus* 15981, kindly provided by Dr Lasa (Valle *et al.*, 2003), and *S. epidermidis* ATCC 35984, were cultured on tryptic soy 5% blood-agar plates (bioMérieux) at 37 °C overnight. Bacteria were then inoculated in tryptic soy broth (bioMérieux), and incubated for 24 h at 37 °C. After this incubation, bacteria were harvested by centrifugation at 3500 g for 10 min at room temperature and washed with PBS (0.15 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , 8.1 mM Na_2HPO_4 , pH 7.4) three times. Subsequently, the bacteria were suspended in PBS and calibrated to a 0.5 McFarland standard confirmed by plate counts.

Bacterial exposure to ceramics and quantification of adhered bacteria.

Bacterial adherence was studied by a protocol adapted from the method of Zamora *et al.* (2007), which evaluated the adherence of similar organisms. The material samples, six of each, were placed in bacterial suspension at 37 °C for 90 min. Following bacterial exposure, the samples were rinsed with sterile PBS three times to wash away any free bacteria and buffer. Subsequently, the samples were placed in 3 ml PBS for 5 min sonication in a low-power bath sonicator, Ultrasons-H 3000840 (JP Selecta). The sonication product was then diluted in PBS and cultivated on blood agar plates to determine the absolute number of bacteria originally adhered to the sample.

Bacterial adherence was tested at pH 7.4 and 6.8. The PBS was acidified by addition of HCl (0.1 M) to the original PBS down to the determined pH. Acidified PBS was subsequently steam-sterilized and stored at 4 °C until experiments were performed.

ζ -potential determination. The concentration of HA and BCP microparticles was 7×10^{-4} g ml^{-1} (4.3×10^8 particles ml^{-1}). The pH of PBS in which these micro particles were suspended was adjusted in the range pH 8 to pH 5 at 25 ± 0.5 °C by the addition of appropriate volumes of 6 M HCl.

The ZetaSizer Nano-ZS (Malvern Instruments) served to measure the size and the electrophoretic mobility of HA and BCP microparticles. This device uses the principle of Brownian motion to measure particle diameter and the principle of laser Doppler velocimetry to measure electrophoretic mobility. The particle diameter and the volume fraction for the HA were 4.74 ± 1 μm and 0.023, respectively; for the BCP they were 4.65 ± 1 μm and 0.026, respectively. The ζ potentials were calculated from their electrophoretic mobility by Smoluchowski's equation. In all cases, the measurements were repeated five times at 25 ± 0.5 °C.

For microbial cells (*S. aureus* and *S. epidermidis*), a ZetaMeter System 3.0+ (ZetaMeter) determined their electrophoretic mobilities by measuring their migration velocity in a direct-current voltage field. The ZetaMeter system possesses a microelectrophoretic cell (type GT-2) and consists of a quartz capillary channel and two reservoirs made of Teflon, with an Mb cylinder anode and a Pt/r rod cathode. The

entire system is hydrodynamically closed. A direct-current power supply (300 V, 50 mA) (Leybold-Heraeus) delivered the voltage between the electrodes, resulting in the determined electric field strength.

Migration times for microbial cells were measured manually with a millisecond timer (Griffin & George). Each individual microbial cell was observed at $6\times$ magnification under a stereo microscope (American Optical), equipped with a micrometer division (with full scale of 160 μm). The typical travel distance for measurements was 40 μm at $25\pm 0.5^\circ\text{C}$. For statistical characterization, the velocity of 10 discrete microbial cells selected at random was measured subsequently at a distance of about 15% of the capillary diameter from the wall to compensate for the cell electro-osmotic flow (Sánchez-Muñoz *et al.*, 2003).

Statistical analysis. The Kolmogorov–Smirnov test served to evaluate the normality of distribution of the data and Student's *t*-test to statistically analyse the results. The criterion for statistical significance was $\alpha=0.05$.

RESULTS AND DISCUSSION

Pore size distribution

Fig. 1 displays the distribution of pore sizes of HA and BCP in the 0.001–10 μm region obtained by mercury intrusion porosimetry measurements after applying the Washburn equation (Washburn, 1921). Both samples investigated exhibited monomodal pore size distribution with a maximum centred at around 0.2 μm . In addition, small secondary pores with sizes $<0.04\ \mu\text{m}$ could be detected whereas pores with diameter $>0.3\ \mu\text{m}$ were not observed. The total porosities were 20% for HA and 50% for BCP.

Bacterial adherence

Bacterial adherence was evaluated by dividing the number of detached bacteria by the external surface area of the sample disc. The number of adhered bacteria varied depending on the material and pH, being in the range

Fig. 1. Pore size distributions of HA (□) and BCP (●) materials obtained by mercury intrusion porosimetry.

3.1×10^3 to 51.9×10^3 c.f.u. mm^{-2} for *S. aureus* and 1.3×10^3 to 24.5×10^3 c.f.u. mm^{-2} for *S. epidermidis*. At pH 7.4 the mean *S. aureus* adherence on HA was 9.38×10^3 c.f.u. mm^{-2} and on BCP 26.4×10^3 c.f.u. mm^{-2} . Moreover, the amount of *S. aureus* adhered varied when adherence tests were carried out at pH 6.8, being 5.0×10^3 c.f.u. mm^{-2} and 10.4×10^3 c.f.u. mm^{-2} , for HA and for BCP, respectively (Fig. 2). For *S. epidermidis*, the mean adherence at pH 7.4 on HA was 17.5×10^3 c.f.u. mm^{-2} and on BCP was 17.8×10^3 c.f.u. mm^{-2} . However, after performance of the adherence assays at pH 6.8, the amount of *S. epidermidis* adhered was 5.2×10^3 c.f.u. mm^{-2} for HA and 10.0×10^3 c.f.u. mm^{-2} for BCP (Fig. 3). The highest adherence occurred in both materials at pH 7.4. The mean adherence of *S. aureus* and *S. epidermidis* on each material decreased 57% when the pH of the PBS medium was reduced from 7.4 to 6.8. *S. aureus* adhered significantly less on HA in both media (Fig. 2). The adherence of *S. epidermidis* on the two materials did not differ statistically, although a tendency toward decreased adherence on HA in the more acidic PBS medium was noted (Fig. 3).

ζ-potential determinations

The ζ potential of the microbial cells was -40.33 ± 4 for *S. aureus* and -44.16 ± 4 for *S. epidermidis* at pH 7.4. At pH 6.8, ζ-potential values slightly decreased, being -38.91 ± 5 for *S. aureus* and -37.43 ± 5 for *S. epidermidis* (Table 1). These negative ζ-potential values agree with results from different strains of *S. aureus* and *S. epidermidis* (Jones *et al.*, 1997; Truesdail *et al.*, 1998; Kim *et al.*, 1999; Wang *et al.*, 2004).

ζ-potential data for HA and BCP microparticles, as a function of pH at a fixed concentration of PBS, were determined (Table 1, Fig. 4). The morphology of these curves is similar to reported data for calcium phosphates (Ducheyne *et al.*, 1992; Yelloji Rao *et al.*, 1993; Borum & Wilson, 2003; Smith *et al.*, 2004). Negative ζ-potential values obtained for these bioceramics, in the pH range tested, range between 6 and 18 mV for HA and between 11 and 21 mV for BCP. The trend observed in ζ potential as a function of pH is similar for both bioceramics, but BCP bioceramic shows a more negative ζ potential than does HA (Fig. 4). The presence of the β-TCP phase in a BCP

Fig. 2. Adherence of *S. aureus* (c.f.u. $\text{mm}^{-2} \pm \text{SEM}$) on BCP and HA at pH 6.8 and 7.4.

Fig. 3. Adherence of *S. epidermidis* (c.f.u. mm⁻² ± SEM) on BCP and HA at pH 6.8 and 7.4.

bioceramic seems, therefore, to increase the net negative surface charge.

Some materials are naturally prone to bacterial adherence because of their chemical or physical characteristics. Basically this seems to be the case with some porous bioceramics used in bone tissue engineering that act as three-dimensional porous scaffolds for natural bone ingrowth (Pereira *et al.*, 2000). The rough and porous surface configuration needed for the ingrowth offers such surface irregularities also to allow bacterial colonization.

With the aim of determining the porosity of HA and BCP, we obtained their pore size distributions by mercury intrusion porosimetry. This analysis disclosed that the smallest pores present in these materials were only 50 nm in diameter; the mean pore size was 200 nm, whereas the maximum pore size was 300 nm. These pore sizes seem too small to allow the accommodation of staphylococci, which exhibit sizes ranging from 0.5 to 1.5 µm (Bannerman & Peacock, 2007). This fact is related to the thick and rigid structure of the peptidoglycan layer of the cell wall of these Gram-positive cocci. Peptidoglycan is a polymer that consists of alternating *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine. These long polymer chains are highly cross-linked to each other via L-alanine, D-glutamic acid, L-lysine, D-alanine tetrapeptides, which bind the NAM residues of adjacent polymer chains to each other. Staphylococcal peptidoglycan layers are much thicker and more highly cross-linked structures than are those in the cell walls of Gram-negative bacteria. This peptidoglycan

cell wall is essential for the survival of staphylococci, as is shown by the bactericidal effect of penicillin derivatives preventing cross-link formation, leading to osmotic rupture of the unprotected bacterial plasma membrane. It is apparent that staphylococci cannot survive without their peptidoglycan layer, but with it they cannot penetrate HA and BCP bioceramics. Due to the rigid structure of the cell wall of Gram-positive bacteria, the small pores present in HA and BCP effectively exclude 0.5–1.5 µm staphylococci from inhabiting the superficial and deep pores. However, if the size of the pores in these materials were larger it would allow bacteria to inhabit the pores, and would weaken the bacterial resistance of the materials and might prevent eradication of adhered bacteria (Becker *et al.*, 2003).

An essential requirement for all bioceramics intended as biomaterial is biocompatibility, i.e. such material must be able to safely fulfil the purpose-designed function under demanding *in vivo* conditions. Interestingly, bacterial adherence studies are usually carried out in PBS or other media in which the pH has been adjusted to the physiological pH of 7.4 supposed to prevail in the extracellular fluid in which the implant and bacteria are immersed. Ample evidence exists, however, that the peri-implant tissues may become acidic due to surgical trauma, aseptic loosening or infection (Bessman *et al.*, 1989; Kontinen *et al.*, 2001). Bioceramics are corrosion resistant, but lowering of the pH might make them more susceptible to bacterial adherence and colonization. Bacterial adherence depends on many factors concerning the biomaterial, including chemistry, hydrophobicity and surface energy, and also depends on bacterial surface properties (An & Friedman, 1998; Brokke *et al.*, 1991). The effect of lowering the pH from 7.4 to 6.8 on staphylococcal adherence was therefore experimentally investigated, because such small pH changes are commonly observed, albeit much more extreme changes are also possible. Although the change seems to be numerically small, it needs to be remembered that this is a logarithmical scale, representing quite a significant change in the concentration of H⁺ ions.

Electrostatic interactions play an important role in microbial cells adsorption on the bioceramic particles. ζ-

Table 1. ζ-potential values (–ζ) and diameter (d) of HA and BCP, and –ζ of *S. aureus* (S. a.) and *S. epidermidis* (S. e.) in 0.2 M PBS

pH	HA		BCP		Bacteria –ζ (mV)	
	d (µm)	–ζ (mV)	d (µm)	–ζ (mV)	S. a.	S. e.
5.5	–	6.00	–	11.40	–	–
6.0	–	8.50	–	13.98	–	–
6.5	–	14.35	–	17.70	–	–
6.8	–	–	–	–	38.91 ± 5	37.43 ± 5
7.0	4.74	16.93	4.65	20.50	–	–
7.4	–	–	–	–	40.33 ± 4	44.16 ± 4
7.5	–	16.63	–	20.46	–	–
8.0	–	18.20	–	21.18	–	–

Fig. 4. ζ potential of HA ($\square$) and BCP ($\blacksquare$) microparticles as a function of pH. The SD values of these results of the electrophoretic mobility studies are indicated.

potential measurements provide a unique method to quantify *in situ* the actual state of the interface between the material and the solution, a state that depends on the polarity of the absorbed ions, the material surface charge, and the ionic concentration in the fluid. When the bioceramics and bacterial cells are dispersed in PBS, an ionic atmosphere with a thickness of 1.5 nm (δ , Debye length) is structured around each material particle and cell (Sánchez-Muñoz *et al.*, 2003). The negativity of the ζ potential of the staphylococcal cell wall, together with that of the ceramic surface, decreases with the drop in pH (Table 1). A decrease in the repulsive forces between substratum and bacterial cell would thus be expected when the pH changes from 7.4 to 6.8; consequently the maximum number of bacteria should adhere at the lower pH value. Somewhat against expectations, however, this lowering of pH did not raise, but significantly reduced adherence of both *S. aureus* (Fig. 2) and *S. epidermidis* (Fig. 3) on both HA and BCP surfaces. This result is surprising, as we know that in infected tissue, where bacterial concentrations can become extremely high, the local pH tends to decrease. This decrease might, however, be a consequence of active bacterial growth more than a prerequisite for it, and actually may not facilitate bacterial adhesion at all. This diminished staphylococcal adherence could be explained by the fact that lowering of pH at some point damages staphylococci and thus impairs their ability to adhere. Our results show that both the surface ζ potential and the number of adsorbed microbial cells were influenced by pH. These behaviours may also be related to the difference in the solubility of the ceramics, and the amounts of Ca^{2+} and PO_4^{3-} ions in their chemical structures. However, as many other factors also affect staphylococcal adherence to the HA or BCP surface, this remains at present only speculation attempting to explain a clear-cut experimental observation.

The present work shows that HA and BCP have sufficiently small pore sizes to exclude staphylococcal bacteria with rigid cell walls. Furthermore, reducing pH from the physiological 7.4 to a slightly acidic 6.8 did not impair this anti-adhesive property, but improved it, so that adherence was diminished quite significantly, by 57 %.

REFERENCES

- An, Y. H. & Friedman, R. J. (1998). Concise review of mechanisms of bacterial adhesion to biomaterial surfaces. *J Biomed Mater Res* **43**, 338–348.
- Bannerman, T. L. & Peacock, S. J. (2007). *Staphylococcus, Micrococcus*, and other catalase-positive cocci. In *Manual of Clinical Microbiology*, 9th edn. Washington, DC: American Society for Microbiology.
- Becker, M. W., Metge, D. W., Collins, S. A., Shapiro, A. M. & Harvey, R. W. (2003). Bacterial transport experiments in fractured crystalline bedrock. *Ground Water* **41**, 682–689.
- Bessman, A. N., Page, J. & Thomas, L. J. (1989). In vivo pH of induced soft-tissue abscesses in diabetic and nondiabetic mice. *Diabetes* **38**, 659–662.
- Bohner, M. (2001). Physical and chemical aspects of calcium phosphates used in spinal surgery. *Eur Spine J* **10**, S114–S121.
- Borum, L. & Wilson, O. C., Jr (2003). Surface modification of hydroxyapatite. Part II. Silica. *Biomaterials* **24**, 3681–3688.
- Bouler, J. M. & Gauthier, O. (1998). Macroporous biphasic calcium phosphates ceramics: Influence of five synthesis parameters on compressive strength. *Biomaterials* **19**, 133–139.
- Brokke, P., Dankert, J., Carballo, J. & Feijen, J. (1991). Adherence of coagulase-negative staphylococci onto polyethylene catheters *in vitro* and *in vivo*: a study on the influence of various plasma proteins. *J Biomater Appl* **5**, 204–226.
- Ducheyne, P., Kim, C. S. & Pollack, S. R. (1992). The effect of phase differences on the time-dependent variation of the zeta potential of hydroxyapatite. *J Biomed Mater Res* **26**, 147–168.
- Gristina, A. G. (1987). Biomaterial-centered infection: microbial adhesion versus tissue integration. *Science* **237**, 1588–1595.
- Hench, L. L. (1991). Bioceramics: from concept to clinic. *J Am Ceram Soc* **74**, 1487–1510.
- Hogt, A. H., Dankert, J. & Feijen, J. (1985). Adhesion of *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* to a hydrophobic biomaterial. *J Gen Microbiol* **131**, 2485–2491.
- Jones, D. S., McGovern, J. G. & Woolfson, A. D. (1997). Role of physiological conditions in the oropharynx on the adherence of respiratory bacterial isolates to endotracheal tube poly(chloride). *Biomaterials* **18**, 503–510.
- Katsikogianni, M. & Missirilis, Y. F. (2004). Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. *Eur Cell Mater* **8**, 37–57.
- Kim, H.-J., Gias, E. L. M. & Jones, M. N. (1999). The adsorption of cationic liposomes to *Staphylococcus aureus* biofilms. *Colloids Surf A Physicochem Eng Asp* **149**, 561–570.
- Kontinen, Y. T., Takagi, M., Mandelin, J., Lassus, J., Salo, J., Ainola, M., Li, T. F., Virtanen, I., Liljestrom, M. & other authors (2001). Acid attack and cathepsin K in bone resorption around total hip replacement prosthesis. *J Bone Miner Res* **16**, 1780–1786.
- LeGeros, R. Z. & LeGeros, J. P. (1996). Calcium phosphate biomaterials in medical application. *Bioceramics* **9**, 7–10.

- Locci, R., Peters, G. & Pulverer, G. (1981).** Microbial colonization of prosthetic devices. III. Adhesion of staphylococci to lumina of intravenous catheters perfused with bacterial suspensions. *Zentralbl Bakteriol Mikrobiol Hyg [B]* **173**, 300–307.
- Pereira, M. A., Alves, M. M., Azeredo, J., Mota, M. & Oliveira, R. (2000).** Influence of physico-chemical properties of porous micro-carriers on the adhesion of an anaerobic consortium. *J Ind Microbiol Biotechnol* **24**, 181–186.
- Pringle, J. H. & Fletcher, M. (1986).** Influence of substratum hydration and adsorbed macromolecules on bacterial attachment to surfaces. *Appl Environ Microbiol* **51**, 1321–1325.
- Reynolds, E. C. & Wong, A. (1983).** Effect of adsorbed protein on hydroxyapatite zeta potential and *Streptococcus mutans* adherence. *Infect Immun* **39**, 1285–1290.
- Sánchez-Muñoz, O. L., Pérez-Hernández, E., Lämmerhofer, M., Linder, W. & Kenndler, E. (2003).** Estimation and comparison of ζ -potentials of silica-based anion-exchange type porous particles for capillary electrochromatography from electrophoretic and electro-osmotic mobility. *Electrophoresis* **24**, 390–398.
- Sánchez-Salcedo, S., Izquierdo-Barba, I., Arcos, D. & Vallet-Regí, M. (2006).** In vitro evaluation of potential calcium phosphate scaffolds for tissue engineering. *Tissue Eng* **12**, 279–290.
- Sari, A., Yavuzer, R., Ayhan, S., Tuncer, S., Latifoglu, O., Atabay, K. & Celebi, M. C. (2003).** Hard tissue augmentation of the mandibular region with hydroxyapatite granules. *J Craniofac Surg* **14**, 919–923.
- Smith, I. O., Baumann, M. J., Obadia, L. & Boulter, J. M. (2004).** Surface potential and osteoblast attraction to calcium phosphate compounds is affected by selected alkaline hydrolysis processing. *J Mater Sci Mater Med* **15**, 841–846.
- Truesdail, S. E., Lukasik, J., Farrah, S. R., Shah, D. O. & Dickinson, R. B. (1998).** Analysis of bacterial deposition on metal hydro(oxide)-coated sand filter media. *J Colloid Interface Sci* **203**, 369–378.
- Valle, J., Toledo-Arana, A., Berasain, C., Ghigo, J. M., Amorena, B., Penades, J. R. & Lasa, I. (2003).** SarA and not σ^B is essential for biofilm development by *Staphylococcus aureus*. *Mol Microbiol* **48**, 1075–1087.
- Vallet-Regí, M. (2001).** Ceramics for medical applications. *Dalton Trans* **2**, 97–108.
- Vallet-Regí, M. (2006).** Revisiting ceramics for medical applications. *Dalton Trans* **44**, 5211–5220.
- Vallet-Regí, M. & González-Calbet, J. (2004).** Calcium phosphates as substitution of bone tissues. *Prog Solid State Chem* **32**, 1–31.
- Wadström, T. (1989).** Molecular aspects of bacterial adhesion, colonization, and development of infections associated with biomaterials. *J Invest Surg* **2**, 353–360.
- Wang, J., Huang, N., Yang, P., Leng, Y. X., Sun, H., Liu, Z. Y. & Chu, P. K. (2004).** The effect of amorphous carbon films deposited on polyethylene terephthalate on bacterial adhesion. *Biomaterials* **25**, 3163–3170.
- Washburn, E. W. (1921).** Note on a method of determining the distribution of pore sizes in a porous material. *Proc Natl Acad Sci U S A* **7**, 115–116.
- Yelloji Rao, M. K., Somasundaran, P., Schilling, K. M., Carson, B. & Ananthapadmanabhan, K. P. (1993).** Bacterial adhesion onto apatite minerals – electrokinetic aspects. *Colloids Surf A Physicochem Eng Asp* **79**, 293–300.
- Zamora, N., Esteban, J., Kinnari, T. J., Celdrán, A., Granizo, J. J. & Zafra, C. (2007).** In vitro evaluation of the adhesion to polypropylene sutures of non-pigmented rapidly growing mycobacteria. *Clin Microbiol Infect* **13**, 902–907.