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Complete Healing of Severe Experimental Osseous Infections Using a Calcium-Deficient Apatite as a Drug-Delivery System

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1. Introduction

1.1 Classifications

Osteomyelitis represents the majority of severe bone infections. The localization of osteomyelitis originating in the bloodstream is most often the metaphysis of long bones [femur (36%), tibia (33%), and humerus (10%)] in children and vertebral bodies in adults (Lazzarini et al., 2004; Calhoun & Manring, 2005) (Figure 1).
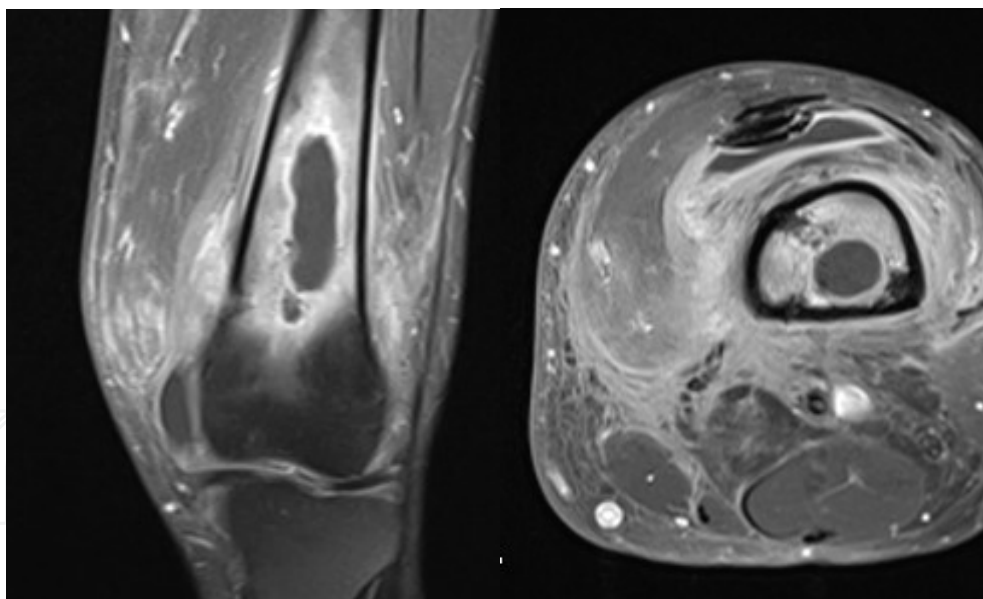

Fig. 1. Magnetic resonance tomography showing an osteolysis of the distal epiphysis of the left femur in an osteomyelitis case in a 40-year-old male patient.

Contiguous-focus bone infections from infected prosthetic devices are more frequently observed in adult males, who are more exposed to trauma (Jorge et al., 2009). Three

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classifications are widely used in clinical practice. Waldvogel et al. (1970) described osteomyelitis according to duration, either acute or chronic. The disease is also classified according to the source of infection (i.e., hematogenous or contiguous focus). A third category defines osteomyelitis in terms of vascular insufficiency (Table 1).

Duration	Origin	Sub-divisions
Acute	Hematogenous	
	Contiguous focus	No generalized vascular disease
		Generalized vascular disease
Chronic	Necrotic bone	

Table 1. Osteomyelitis staging system (adapted from Waldvogel et al., 1970)

Nosocomial or traumatic transmissions are not considered in this classification system. Cunha (2002) suggested a simple classification based on acuteness (acute, subacute, or chronic) and microorganisms present for the elderly. The most recent classification system, based on anatomical, clinical, and radiologic features, was described by Cierny et al. (1985) (Table 2). Osteomyelitis is first defined by four stages, depending on the degree of extension: confined (medullary bone), superficial (cortical bone), localized (both), and diffuse. Second, the status of the patient (host) is considered, from A (healthy) to C (severely compromised).

Stages	Anatomical type	Description
1	Medullar	Endosteal focus
2	Superficial	Limited to the surface of the bone
3	Localized	Full thickness of the cortical bone
4	Diffused	Entire cortical bone is involved
Host	Description	Equivalent ASA status
A	Normal	ASA 1
B	Bs: systemic compromise	ASA 2 - 4
	Bl: local compromise	
	Bsl: local and systemic compromise	
C	Major comorbidities	ASA 5 -6

Table 2. Adult osteomyelitis staging system (adapted from Cierny et al., 1985)

1.2 Microbiology
In the case of acute osteomyelitis (AHO), the infection is caused by a hematogenous pathogen and is often located at a metaphysis (Table 3). *Staphylococcus aureus* is isolated in 60-80% of cases (Gafur et al., 2008) with an increasing minimal inhibitory concentration (MIC) to methicillin, followed by other Gram-positive cocci (i.e., coagulase-negative staphylococci, *Streptococcus* spp.), *Pseudomonas aeruginosa*, and *Escherichia coli* (Lew & Waldvogel, 1997; Gutierrez, 2005; Saavedra-Lozano et al., 2008) (Table 4). Bacteria are isolated from blood cultures or tissue biopsy in only 45% in children. Incidence of osteomyelitis was found to reach one in 808 to more than 2,000 admissions (Georgens et al., 2004; Weichert et al., 2008). Methicillin-resistant *S. aureus* (MRSA) represents approximately 10% of the causative bacteria, except in the USA (40-50%) (Weichert et al., 2008). The

presence of Panton-Valentine leukocidin could explain the persistence and rapid local extension of AHO in humans (Crémieux et al., 2009; Labbé et al., 2010).

< 1 year	1 to 16 years	More than 16 years
Group B streptococci	*Staphylococcus aureus*	*Staphylococcus epidermidis*
Staphylococcus aureus	*Streptococcus pyogenes*	*Staphylococcus aureus*
Escherichia coli	*Haemophilus influenzae*	*Pseudomonas aeruginosa*
		Serratia marcescens
		Escherichia coli

Table 3. Organisms commonly isolated in osteomyelitis based on patient age (adapted from Dirschl et al., 1993)

Bacteria	Circumstances
Staphylococcus aureus	All types of osteomyelitis
Coagulase-negative staphylococci or *Propionibacterium* species	Foreign-body–associated infection
Enterobacteriaceae sp or *Pseudomonas aeruginosa*	Nosocomial infections
Streptococci or anaerobic bacteria	Wounds infected by saliva, diabetic foot lesions, decubitus ulcers
Salmonella species or *Streptococcus pneumoniae*	Sickle cell disease
Bartonella henselae, *Aspergillus* sp, *Mycobacterium avium*-intracellulare or *Candida albicans*	Immunocompromised patients
Pasteurella multocida or *Eikenella corrodens*	Bites

Table 4. Main bacteria isolated in bacterial osteomyelitis (adapted from Lew et al., 1997)

1.3 Diagnosis and treatment

The clinical picture of AHO is often classic: it is commonly a child from 6 to 12 years of age who suddenly presents with a disability absolved from the affected limb and associated with a 39-40°C fever. After clinical investigation, the pain is extremely intense, and the preferential location is the lower extremity of the thighbone or the superior extremity of the shin. Emergency treatment must be initiated, associated with fixed immobilization in plaster and intravenous antibiotic therapy. If the treatment is administrated early, cure is most often achieved within 3 weeks. If a delay in diagnosis is made or the treatment is inadequate, chronic osteomyelitis associated with a 38°C fever can develop and the affected limb aches, is red and warm, and sometimes abscesses. Radiography shows osteolysis at the metaphysis, with thickening or detachment of the periostium with ossification and appearance of a sequestrum. The treatment of these forms, which can become subacute or chronic, is complex and is often associated with 6 months to 1 year of medical treatment and repeated surgical procedures to remove intra-osseous or under-periostium abscesses and sequestra. The after-effects are important: osseous fragility with risk of fracture and disorders of healing and growth in length or with deviation. The osteitis is usually subacute with *S. aureus* and more or less painful. When osteitis affects a lower limb, patients have a

limp, and clinical examination shows amyotrophy regarding the skeletal area of interest. The biology shows all the signs of infection (leucocytosis, erythrocyte sedimentation rate, C-reactive protein); radiology reveals evidence of uni- or polycyclic osteolysis that is finely encircled by dense bone (Brodie's abscess). Surgical treatment is necessary, and osseous trepanning is performed to decompress this internal infection, administering antibiotics intravenously first and then orally. The duration of treatment is at least 3 weeks. If everything does not quickly normalize, oral antibiotics are necessary for several months. Osteomyelitis is an infective process that may also require numerous surgical interventions and leads to bone sclerosis and deformity, and even to a loss of limb.

2. Animal experimental models

Clinical trials for antibiotic treatment of osteomyelitis are rare and difficult to perform for many reasons. First, the anatomical localization of the lesions varies. Moreover, the treatment of patients suffering from severe bacterial infection with new drugs raises complicated ethical concerns that must be addressed. In vitro alternatives to replace animal tests, specifically to study osteomyelitis due to MRSA, are unrealistic and do not allow for surgical procedures. For these reasons, animal studies are the most appropriate and feasible way to assess the impact of antibiotic therapy on the outcome of osteomyelitis. Several different models exist and were developed to study hematogenous (or post-traumatic) osteomyelitis or osteomyelitis related to orthopedic implants and prosthetic joint infections. The features and history of these osteomyelitis models have been summarized by several authors (Mader, 1985; Norden, 1988; Rissing, 1990; Patel et al. 2009). Further, osteomyelitis studies have been conducted using various species, including rats (Power et al. 1990), rabbits (Andriole et al., 1973; Norden 1988), dogs (Deysine et al., 1976; Fitzgerald, 1983), and guinea pigs (Passl et al., 1984)., each with advantages and limitations.

2.1 Limitations and failures

The osteomyelitis experimental model is demanding but critical for testing new antibiotics because eradication of bacteria from bone represents a very difficult challenge (Yin et al., 2005). In experimental studies, viable bacteria can be retrieved from the bone despite prolonged antibiotic treatment (i.e., up to 4 weeks). The development and maturation of bacterial biofilms could explain the failure of antibiotic treatments and subsequent relapses (Brady et al. 2008). However, current animal models of osteomyelitis have a number of limitations, including low success rates for the induction of osteomyelitis, the elimination of causative bacteria by the host immune system, and the need for administration of sclerosing agents (SAs) in most cases (Patel et al., 2009). These SAs, as morrhuate sodium or its derivative arachidonic acid, are thought to induce varying degrees of aseptic bone necrosis, providing ideal conditions for bacterial proliferation and likely facilitating bone infection by occluding the microvasculature. In most animal models of osteomyelitis, SAs are usually injected prior to bacterial injection (Yoshii et al., 2001; Fukushima et al., 2005).

2.2 The acute rabbit model

In the acute model developed by Gaudin et al. (2011), devascularized bone made from a surgically induced bone defect provided a site in which to establish a productive infection.

Femoral trepanation using a biopsy needle was followed by injection of 1 mL of 10^9 colony-forming units (CFU)/mL *S. aureus* suspension directly into the knee cavity. Using this protocol, bacterial densities approached 9-$\log_{10}$ CFU/g infected tissue 3 days post-infection that persisted at least 14 days without treatment. Unlike chronic models of osteomyelitis, no spontaneous recovery of the bacterial infection was observed. Moreover, the rabbit long bone model is appropriate for the study of osteomyelitis because rabbits are more prone to infection than other animals, such as rats. The size of New Zealand white rabbits makes it possible to more closely mimic human surgical procedures such as bone debridement and computer-controlled pharmacokinetic .
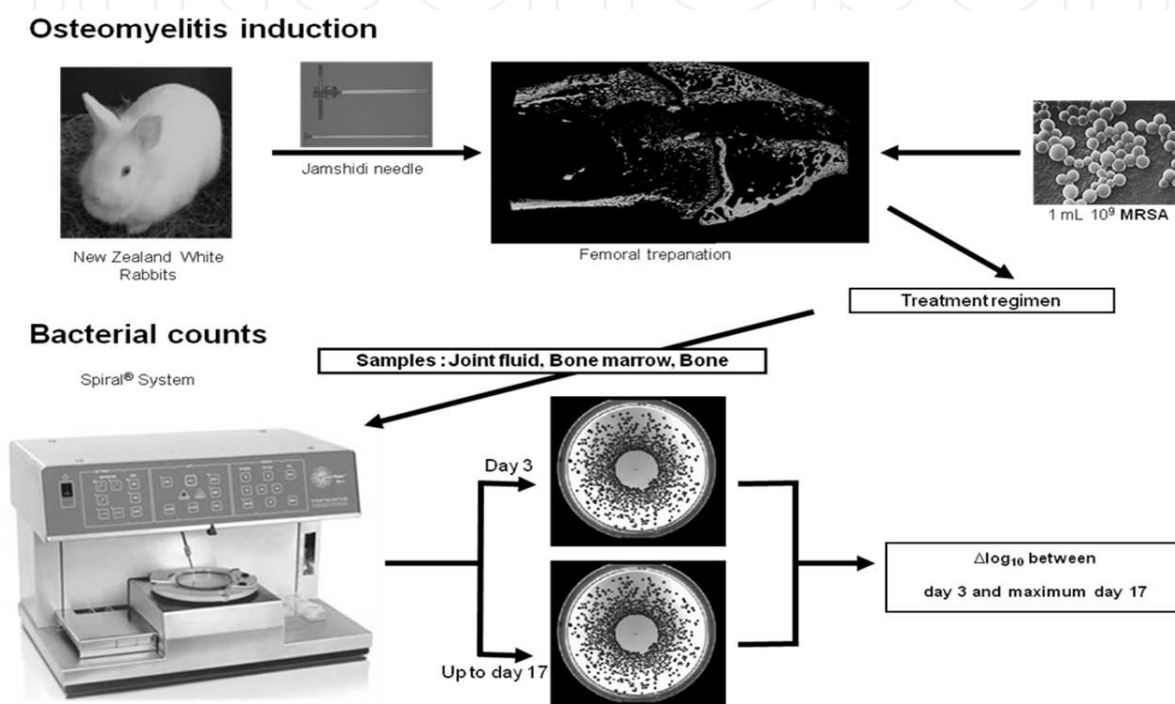

Fig. 2. The acute experimental osteomyelitis rabbit model (Gaudin et al., 2011)

3. Calcium phosphate as a matrix of antibiotic release

The term "biomaterial" has recently been defined as "a substance that has been engineered to take a form that, alone or as part of a complex system, is used to direct…the course of any therapeutic or diagnostic procedure" (Williams, 2009). Implantable biomaterials are inert or can promote biological activities, such as bone regeneration, or minimize undesirable activities, such as infection or blood clotting (Williams, 2008, 2009).

In the osseous and dental fields, biomaterials are often necessary to fill or treat different pathological situations, such as bone trauma, infections, irradiations, or various diseases such as osteoporosis and tumor resection (Campoccia et al., 2010). As alternatives to bone grafts, different biomaterials have been developed.

3.1 Biomaterials

Inorganic materials are frequently used as bone matrixes and are divided into three chemical families that represent current alternatives to biological bone grafts: calcium

phosphate (CaP), calcium sulphate, and calcium carbonate. These materials can be shaped into different forms such as powders, granules, ceramics, cements, and coating, depending on the site, and the size and shape of the bone defect. Granules are more convenient than blocks, allowing the replacement of a large bone volume. As blocks are difficult to fit into cavities, vacant areas are often observed between blocks and bone. Vertebrate bone tissue is primarily composed of CaP, which explains why CaP materials are excellent candidates for bone reconstruction (Rush, 2005; Vallet-Regi, 2006: LeGeros, 1991). CaP materials are also interesting because of their cell resorption and osteoconductive properties. Calcium sulphate and calcium carbonate are less frequently used because they promote poor osseous formation because of their higher solubilities.

Based on composition, currently used synthetic CaP matrixes are classified as hydroxyapatite [HA: $Ca_{10}(PO_4)_6(OH)_2$], alpha- or beta-tricalcium phosphate [α or β-TCP : $Ca_3(PO_4)_2$], mixtures of these compounds, biphasic CaP (BCP), or unsintered apatites called calcium-deficient apatite (CDA).

HA and ß-TCP ceramics can be prepared by grinding CaO and P_2O_5 powders with Ca/P equal to 1.67 and 1.5, respectively. These mixtures must be sintered at more than 1100°C. These CaP biomaterials differ in their extent of dissolution (Chow, 2009):

$$\alpha\text{-TCP} >> \text{CDA} > \beta\text{-TCP} >> \text{HA}$$
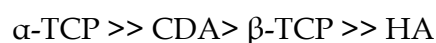
CDAs can be prepared either by aqueous precipitation from calcium and phosphate salts or alkaline hydrolysis of acidic CaP (Jarcho, 1981; Gauthier et al., 1998; Venesmaa et al., 2001; Nehme et al., 2003). For BCPs, the dissolution rate depends on the HA/TCP ratio: the higher the ratio, the higher the dissolution (LeGeros, 1991; Daculsi et al., 1997). HA, TCP, BCPs, and CDA are frequently described in the literature as excellent candidates for bone substitution because of their similarity to bone structures (Figures 3 and 4).
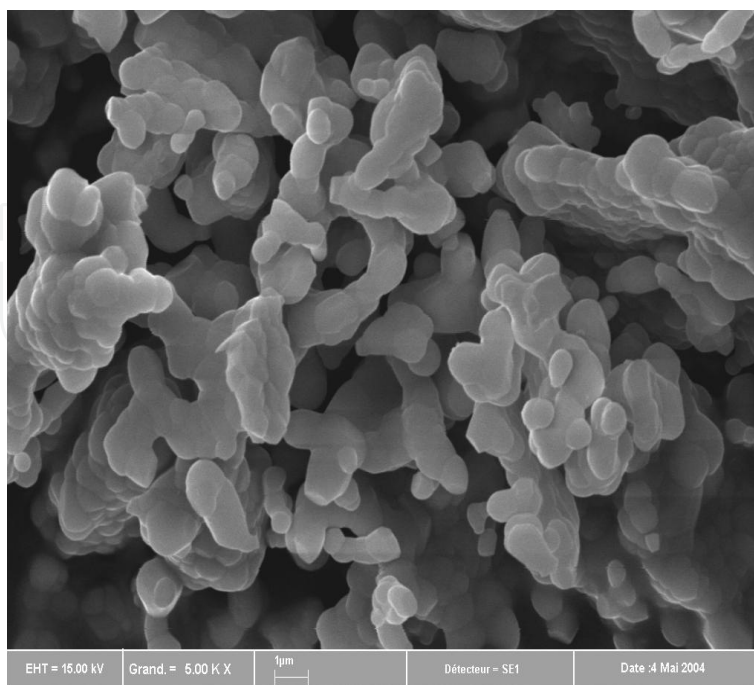

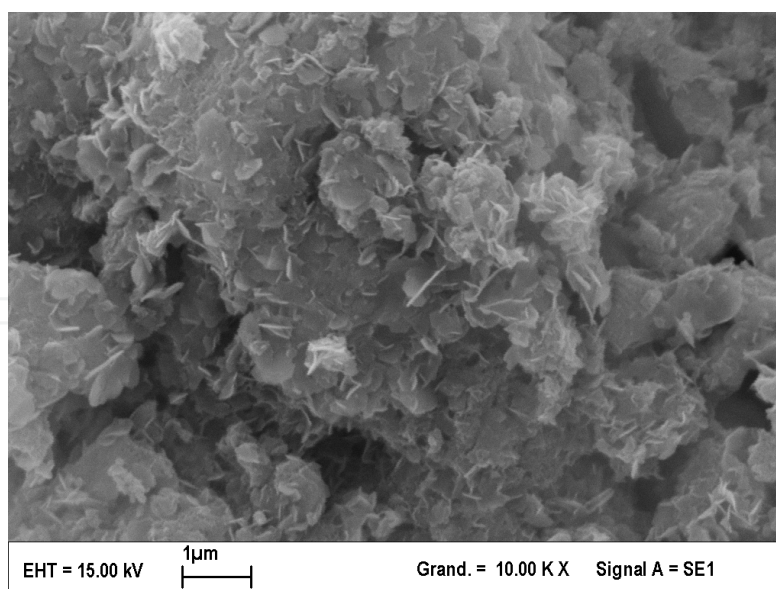Fig. 3. Scanning electron micrograph picture of BCP at a magnification of 5000.

Fig. 4. Scanning electron microscopy picture of CDA at a magnification of x10 000.

These materials have all the necessary properties required for a graft: biocompatibility, bioactivity, biofunctionality, and osteoconductivity. Because of CDA's better solubility than BCP, bone colonization with CDA will be quicker and more significant and will thus provide a better reconstitution of the bone.

Others matrix properties are also very important. Macroporosity, which corresponds to pores larger than 100 μm, defines its capacity to be colonized by cells. Different agents can be associated with the matrix during the preparation process (naphthalene or sucrose particles and granules) and then calcinated or sublimated at high temperature (Lecomte et al., 2008; Le Ray et al.; 2010). Microporosity, corresponding to pores smaller than 10 μm, defines the matrix capacity to be impregnated by biological fluids. These micropores depend on the sintering process, and the microporosity depends mainly on the material composition and the used thermal cycle. Solubility and biological properties of these CaP materials depend on crystal size, ionic impurities, specific surface area, and porosity. The control of the macro- and micropore size and distribution of CaP bone substitutes represent the most important parameters to promote or induce bone formation. All these parameters have a specific influence on the final mechanical properties of the bioceramics (Bouler et al., 1996).

CaP biomaterials possess three fundamental properties that govern potential bone substitution:

- Biocompatibility: CaP ceramics are perfectly tolerated by the host organism, as described by numerous studies (Deligianni et al., 2001; Ooms et al., 2003; Julien et al., 2007; Williams, 2008),

- Bioactivity: After implantation, the biological fluids interact with the CaP ceramics and initiate the dissolution of the material. Depending on the chemical composition of the CaP ceramics, a precipitation of a layer of biological apatite can be obtained on their surfaces. The continuity thus obtained between the host bone tissue and the biomaterial promotes cellular colonization and the formation of bone tissue. The cellular resorption and degradation of the bone substitute results from the concomitant action of osteoclasts and macrophages, respectively (Anderson & Miller, 1984; Minkin & Marinho, 1999; Detsch et al., 2008). The resorption rate of the material and the *de novo*

bone tissue rate ideally must be similar to ensure stability of the interface (Zerbo et al., 2005). The biological, chemical, and mechanical properties at the bone/material interface are therefore essential to ensure good osteointegration of the implant (Ducheyne & Cuckler, 1992).

-   Biofunctionality (Daculsi et al., 1999; Parikh, 2002; LeGeros, 2002): The material mechanical properties of CaP ceramics are limited by low initial mechanical properties compared to host bone mechanical properties. However if osteoconduction and resorption are favored (e.g., by a convenient porous structure), the fragility of the CaP ceramic implant is going to decrease, and an optimal final biofunctionality will be achieved with the total resorption/substitution process. Therefore, intrinsic material parameters [e.g., rate of porosity and solubility (Ca/P ratio)] and extrinsic parameters (e.g., primary stability, instrumentation) must be adapted to promote both complete implant resorption and tissue regeneration. Then, the three functions (mechanic, metabolic, and hematopoietic) of bone can be fully restored.

3.2 Biomaterials as drug delivery systems

The "fill-in" properties are interesting but rapidly it became necessary to not only fill in osseous or dental defects (Navarro et al., 2008) but to treat locally different pathologies. Thus, several combinations of biomaterial matrices and therapeutic agents were prepared. Such biomaterials are called drug delivery systems (DDS). First, polymethylmethacrylate cements were tried as innovative drug delivery systems, but these materials were progressively replaced by resorbable materials whose major advantage is to be left *in situ* in the bone defect and do not require surgical removal. These materials are numerous, ranging from inorganic to organic and from natural to semi-synthetic or synthetic.

Therapeutic agent-CaP biomaterial combinations prepared to produce an *in situ* DDS with a sustained release profile were numerous; a large panel of therapeutic agents loaded onto biomaterials was used, including growth factors (Verron et al. 2010) such as bone morphogenetic proteins (Ripamonti et al., 1992; Deckers et al., 2002), human growth hormone (Goodwin et al., 1995; Downes et al., 1995; Guicheux et al., 1998), transforming growth factor-beta (Kim et al., 2005), insulin-like growth factor (Matsuda et al., 1992); antiosteoporotics (Denissen et al., 1994, 1997; Golomb et al.,1992); anticancer drugs (Otsuka et al., 1995; Itokazu et al., 1998); insulin (Otsuka et al., 1994); steroid hormones (Bajpai & Benghuzzi, 1988); analgesic drugs (morphine and lidocaine (Gautier et al., 2010), and antibiotics (Penner et al., 1996; Suzuki et al., 1998).

Concerning antibiotics, the incorporation of antibacterial therapeutic agents in biomaterials dates back to the 1950s with the association of dental cements and resins with antibiotic drugs. The idea was to release locally therapeutic agent at the infection site. As cements and resins are not bioresorbable, other biomaterials that are resorbable and soluble were developed. Therapeutic agent release must be controlled to ensure adequate tissue concentrations several times higher than the MIC and maintained sufficiently to entirely cover the difficult post-surgery period, avoiding the systemic administration of intravenous or oral antibiotics and their subsequent side effects. Among the antibiotics (Sudo et al., 2008), gentamicin sulphate (Specht & Kühn, 1998) and crobefat (Joschek et al., 1998), cephalexin (Yu et al., 1992), tobramycin (Nijhof et al., 1997; Anaja et al., 2008), arbekacin (Itokazu et al., 1997), ciprofloxacin ((Wu et al., 1997), isepamicin sulphate (Itokazu et al., 1998; Kawanabe et al., 1998), gentamicin (Randelli et al., 2010), and vancomycin (Hamanishi et al., 1996) are commonly used for these associations. Recently, a new DDS using linezolid

was developed (Gautier et al., 2010). Tetracyclines cannot be used with CaPs because their fixation to the matrix is irreversible (Misra, 1991). Tetracycline is moreover used as a tracer for forehead mineralization in histology studies but is not used for the treatment of children.

3.3 Associating therapeutic agents with biomaterials

Various techniques associating a therapeutic agent with a CaP biomaterial have been reported in the literature: powder-powder mixing (Yu et al., 1992; Dacquet et al., 1992; Hamanishi et al., 1996; Trécant et al., 1997); soaking of beads (Thomazeau & Langlais, 1996; Brouard et al., 1997), granules (Joschek et al., 1998), or blocks (Prat-Poiret et al., 1996) in a therapeutic agent solution; packing the therapeutic agent in a central, cylindrical cavity in porous blocks (Shinto et al., 1992) or in the central cavity of TCP capsules to maintain high-level, long-term release of the therapeutic agent (Wu et al., 1997); adsorption of the therapeutic agent in solution on the biomaterial (Guicheux et al., 1997; Trécant et al., 1997; Gautier et al., 1998); centrifugation (Itokazu et al., 1995; Nijhof et al., 1997; Itokazu et al., 1994a, 1998d, 1998e, 1998f); or immersion of a biomaterial block in a therapeutic agent solution followed by vacuum (Itokazu et al., 1998f; Kawanabe et al., 1998). Adsorption and soaking both allow the therapeutic agent to be incorporated at the surface of the biomaterial, whereas centrifugation and vacuum enable the therapeutic agent to enter the pores of the biomaterial. These different processes of therapeutic agent–matrix association are chosen to either facilitate contacts between the biomaterial and the therapeutic agent or achieve compaction. At the same time, our laboratory has dismissed the use of wet granulation and developed two compaction techniques: dynamic compaction and isostatic compression for the successful preparation of sustained-release forms.

The technique of wet granulation, a densification technique widely used in the pharmaceutical industry for the manufacture of granules and pellets, is commonly used for the association of CaP (CDA or BCP) with the therapeutic agent, making it possible to acquire a homogeneous distribution of the constituents of the granules and create close links between CDA and the therapeutic agent (Ormos, 1994). In addition, the acquired granules have a spherical form that is suitable for filling bone defects. This technique has already been used for bone substitute formulations with vancomycin and linezolid (Gautier H et al., 2000). A particle size analysis by laser light diffraction can be performed on acquired granules after sieving of the fraction (40- to 80-µm fraction, 80- to 200-µm fraction, 200- to 500-µm fraction, depending on the defect size). The results show that a large majority of the granules belong to the required fraction. If necessary, and to select more precisely the fraction desired, aspiration with an air jet sieving machine can be performed.

Dynamic compaction is a powder compaction technique developed in 1995 to consolidate CaP powders (Trécant et al., 1997). During this process, particle surfaces are highly deformed, producing interparticulate bonding in a one-step procedure. This process occurs during the passage of a shock wave through the powder. As this technique requires no external heat and allows the compaction to be formed without a sintering step, a heat-sensitive therapeutic agent can be associated with a CaP powder without denaturing the active element. The agent and powder can be melted and associated before compaction, and the pressure can vary between 0.5 and 2 MPa. Different studies showed the advantage of dynamic compaction to obtain compact CaP biomaterials (Trécant et al., 1995) and to associate therapeutic agents with those materials (Guicheux et al., 1997; Trécant et al., 1997). These studies investigated the association of growth factors (e.g., human growth hormone) and antibiotics, such as vancomycin and polymyxin B, with the CaP matrix (CDA and BCP).

The physicochemical characterization of CaP granules by X-ray diffraction, infrared spectroscopy, and nuclear magnetic resonance showed that the structures of BCP and vancomycin were unchanged by dynamic compaction at 1.9 MPa. This finding was concordant with another study (Trécant et al., 1995), showing that the structures of powders such as hydroxyapatite, β-CaP, BCP, and octacalcium phosphate were conserved after a 2-MPa dynamic compaction. Scanning electron microscopy showed that granule porosity depends on the manufacturing process, ranging between 37.7 ± 6.8 and 9.9 ± 4.7%. Granule porosity with dynamic compaction was 3-to 4-fold lower than with wet granulation. In fact, the wet granulation process is performed during a single step in which densification occurs, whereas granule preparation is done in two steps with the dynamic compaction process: densification by powder volume reduction (which gives a compact with lower density) followed by crushing. This volume reduction is correlated with the pressure applied (porosity is reduced when compaction pressure is high). As bone ingrowth correlates with material porosity (Lu et al., 1999), the choice of preparation process allows various granules to be obtained.

Polymyxin B, a polypeptidic antibiotic that undergoes thermodamage above 60°C, was also studied and associated with CaP by dynamic compaction (Kimakhe et al., 1999). The biological activity of polymyxin B-loaded CaP was determined by the effect of the antibiotic and monocyte/macrophage degradation on compact surfaces. The biological activities (i.e., antibacterial activity and inhibited lipopolysaccharide effects on monocyte/macrophage CaP degradation) of polymyxin B released from compacted calcium-deficient apatite were unaltered. Thus, dynamic compaction allows polymyxin B to be used with CaP ceramics without any loss in integrity or biological effects.

Isostatic compression is a technique based on the transmission of an isostatic omni-directionnal hydraulic pressure to powder, thus making the materials denser within a few minutes at room temperature. This cold technique allows the association of drugs without any degradation and allows the direct preparation of a correctly and directly molded implant. Compression pressure is applied uniformly and from all directions to consolidate the material (Gautier et al., 2000a, 2000b).

Physicochemical characterization of BCP granules by X-ray diffraction, infrared spectroscopy, and nuclear magnetic resonance showed that BCP, linezolid, and vancomycin structures remained the same. After association, release profiles must be determined to characterize the biomaterials. To establish the granule release profiles of the therapeutic agents, different tests can be used: culture chamber dissolution tests (Guicheux et al., 1997) or paddle apparatus dissolution tests (European Pharmacopoeia 7.1). Proportions of therapeutic agents released daily from a CaP matrix must be measured by an UV–visible spectrophotometric or high-pressure liquid chromatography (HPLC) assay.

Generally, using the culture chamber dissolution test, independent of the therapeutic agent (e.g., vancomycin or linezolid), after a waiting time of approximately 6 hours, the kinetics were observed to be order 0 up to a complete release in 3 to 26 days, depending on the amount of therapeutic agent loaded, its solubility, and the process of association. The waiting time of 6 hours, noticed before the beginning of the release of the antibiotic, corresponds to the time of the anchoring of grains, necessary at the beginning of the dissolution of the antibiotic in the medium.

For example, in wet granulation, the process allows a faster delivery, releasing the associated vancomycin in a maximum of 3 days. Dynamic compaction increases the period of vancomycin release to 4 to 6 days. Granules obtained by this process form a matrix that releases the therapeutic agent slowly, depending on the binding force (not yet determined)

of vancomycin to CaP. The pressure of dynamic compaction has no significant influence on release time, and there was no significant difference in the vancomycin adsorbed on granules prepared by dynamic compaction or compacted with BCP granules. In the first case, the water of the dissolution medium penetrates into the pores to release the therapeutic agent; in the second case, a vancomycin dissolution-diffusion process operates from the periphery toward the center of the granule.
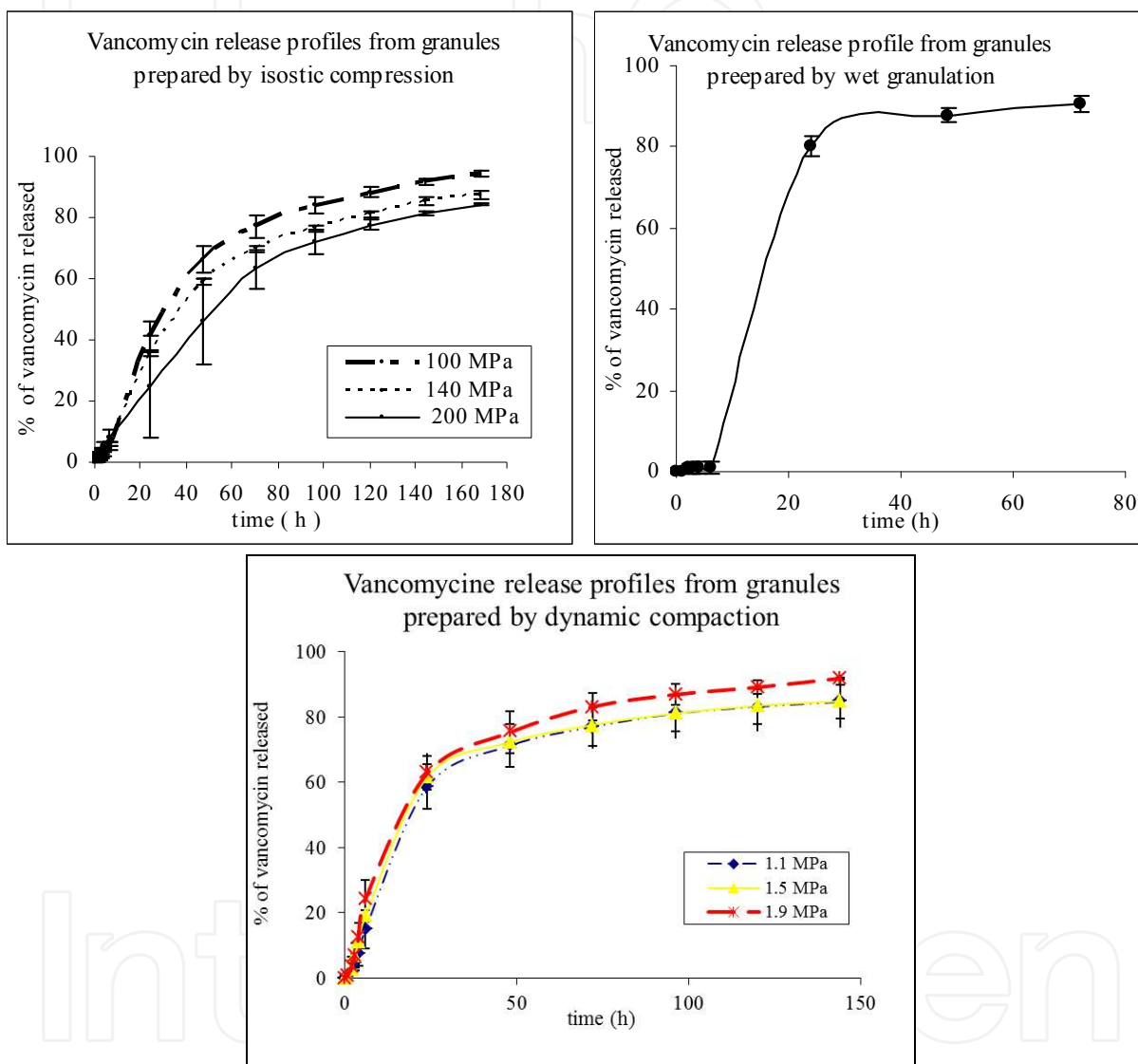

Fig. 5. Vancomycin release profiles from BCP granules prepared by different techniques.

Another study compared wet granulation and isostatic compression (Gautier et al., 2000b). Release was faster for granules prepared by wet granulation than for those prepared by isostatic compression. Moreover, vancomycin release time was prolonged as compression increased: an increase in isostatic compression from 100 to 200 MPa allowed a doubling of 75% vancomycin release. The use of isostatic compression allowed a 3- to 5-fold increase in the period of vancomycin release compared to granules prepared by wet granulation. These differences in the rate of vancomycin release may have been due to the nature of BCP-vancomycin binding strength. Vancomycin is always released faster when associated by

adsorption at the surface of the granule compared to direct incorporation into the granule mass, regardless of the manufacturing process used. Release is also faster for wet granulation than for isostatic compression. Moreover, therapeutic agent release slows as isostatic pressure increases. Figure 5 shows different vancomycin release profiles from 200- to 500-μm BCP granules prepared by isostatic compression, wet granulation, and dynamic compaction.
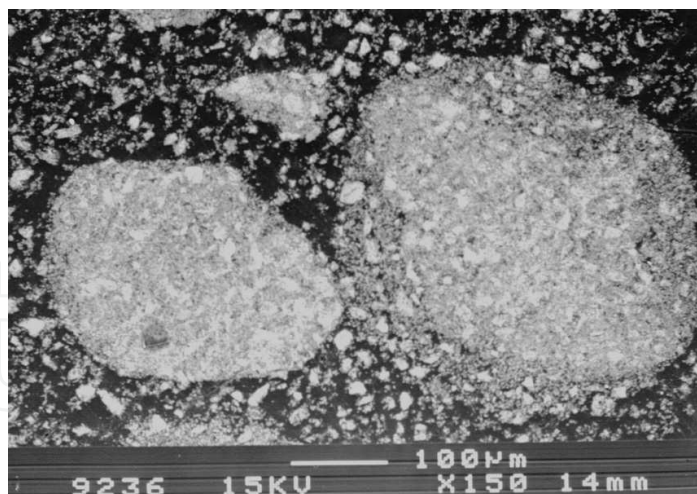
Scanning electron micrographs show that granules prepared by wet granulation have a greater number of macropores and those prepared by isostatic compression possess a greater number of micropores (Figure 6). Image analysis also indicates that porosity is greater for granules prepared by isostatic compression. Pores of granules prepared by wet granulation appear to be more accessible to the release medium, thereby increasing the rate of vancomycin release. Moreover, the porosity percentage for granules prepared by isostatic compression is greater as isostatic pressure increases.
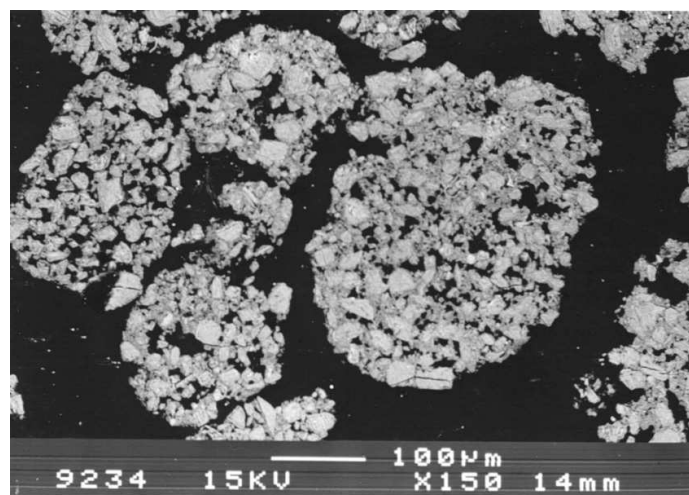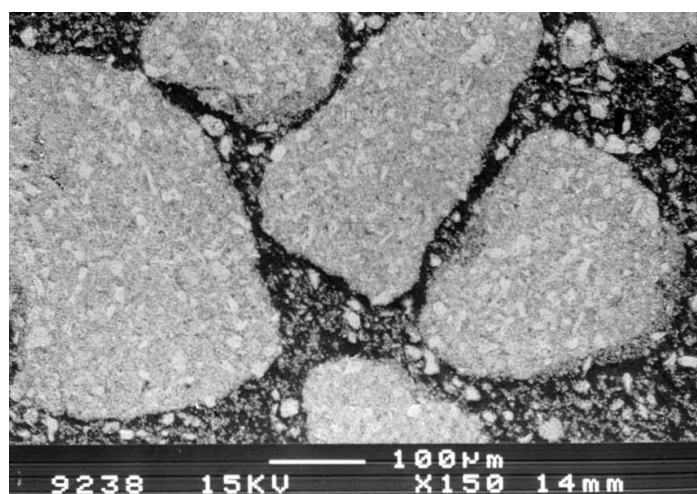

6a: porosity = 33.93 ± 4.29 %
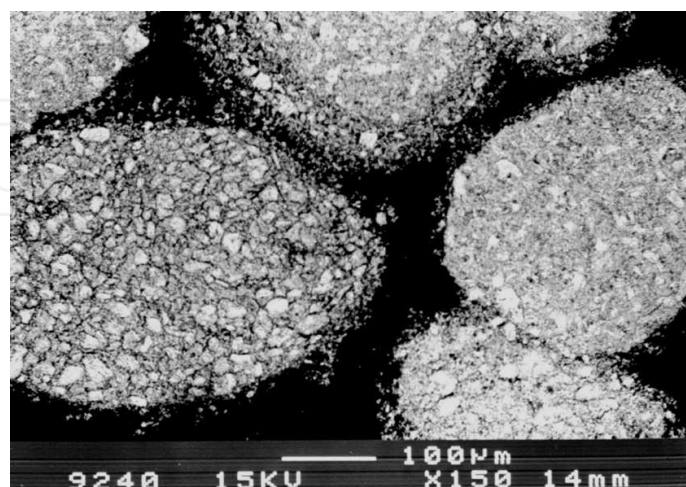
6c: porosity = 47.92 ± 5.94 %

6b

porosity = 63.4 ± 14.30 %

6d

porosity = 42.71 ± 7.48 %

Fig. 6. Scanning electron micrographs of BCP granules. BCP granules (200- to 500-µm) were prepared by wet granulation (a) and isostatic compression at 100 MPa (b), 140 MPa (c), and 200 MPa (d). Porosity percentages are expressed as the mean ± SD (Gautier et al., 2000b)

Another study was performed on CDA granules containing linezolid associated by a wet granulation process (Gautier H, Biomaterials 2010). Figure 5 shows images of CDA containing 10% linezolid (a) and 50% linezolid (b) acquired by scanning electronic microscopy. A comparison of the slope of the linear regression was made. For 10% linezolid-loaded granules, the slope (p) was equal to 17.068 with correlation coefficient $r^2 = 0.994$; for 50% linezolid-loaded granules, p = 5.11 and $r^2 = 0.9969$. For granules containing 10% linezolid, the release was rather quick (3.3 times more important for 10%-loaded granules than for 50%-loaded granules). After a waiting time of approximately 6 hours, the kinetics observed were order 0 up to a complete release in 9 days. The results of the release kinetics for CDA loaded with 10% linezolid were similar to those observed for vancomycin integrated into BCP, although with a slightly longer release time. For the release kinetics of CDA containing 50% linezolid, the release kinetics were similar, with a waiting time from

approximately 6 hours, and kinetics of order 0. The release was complete after 26 days, which is three times greater than for grains loaded with 10% linezolid.
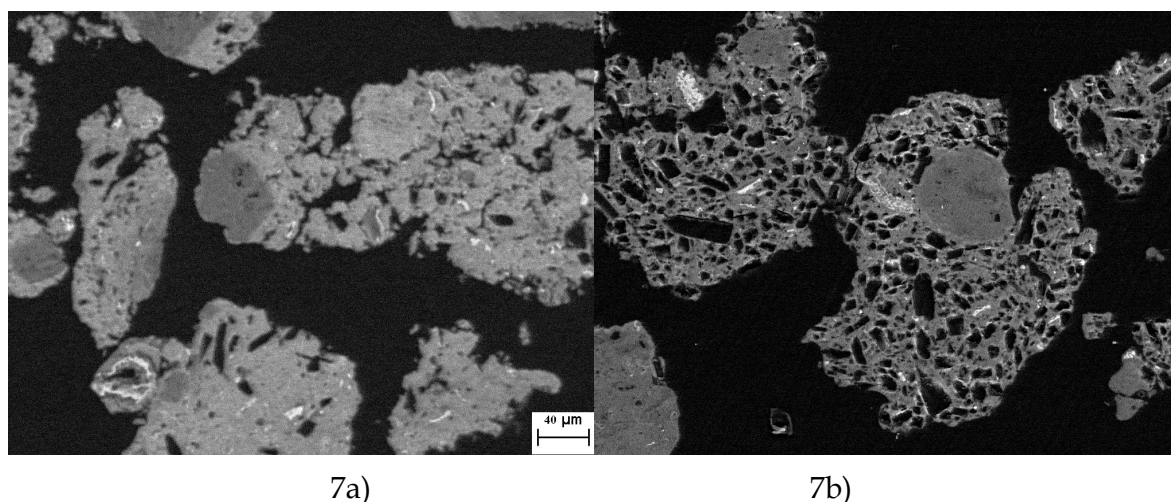

7a)   7b)

Fig. 7. CDA containing 10% linezolid (a) and 50% linezolid (b) acquired by scanning electronic microscopy at a magnification of ×250 (Gautier et al., 2010a)

Another laboratory (Kundu et al., 2010) developed HA-based porous scaffolds loaded with ceftriaxone-sulbactam (i.e., a drug combination consisting of an irreversible β-lactamase inhibitor and a β-lactam antibiotic) and presented that results of in vitro and in vivo drug elution after 41 days showed release rates higher than minimum inhibitory concentration of ceftriaxone-sulbactam against *S. aureus* in a chronic osteomyelitis model.

Additionally, there is no correlation between these in vitro release results, the release test used, and the in vivo therapeutic agent released. It appears to be important to choose the fabrication process in terms of the needed release time: a flash release of just a few days to avoid infections after surgical intervention or long-term release at high concentrations after bone infection.

It is important to demonstrate the effectiveness of the antibiotic released from the granules. This can be performed by nuclear magnetic resonance-mediated structural identification as well as by antibacterial assay. The analysis by ^{1}H- nuclear magnetic resonance (^{1}H-NMR) must be performed on samples of CaP granules to verify that the association process does not modify the CaP structure. ^{1}H-NMR analysis must also be performed on samples of CaP granules loaded with therapeutic agent to identify the structure of the therapeutic agent molecule in the different samples regardless of the percentage of therapeutic agent associated. Analysis performed on BCP and CDA showed that wet granulation, isostatic compression, and dynamic compaction processes did not affect the structure of the CaP matrices. Analysis performed either on vancomycin or linezolid showed that the incorporation of pharmacological agents into CDA and BCP by wet granulation and isostatic compression did not affect the structure of the antibiotic. The chemical structure of the vancomycin and linezolid remained identical in granules even during release. In fact, no significant spectral differences were noticed during the NMR analyses of the therapeutic agent molecule released and extracted from the release profiles from 24 hours to 7 days.

The bacteriological test consists of measuring the amount of therapeutic agent still active in samples after its release. Bacterial strains must be shown to be sensitive to the therapeutic

agent, and the matrices (i.e., CDA and BCP) do not inhibit the bacterial growth of the bacterial strain. The ratio of the therapeutic agent (i.e., vancomycin or linezolid) quantities calculated by bacteriological and assay measurements, derived from release kinetics of the granules after 24 hours, 5 days, or 7 days of release are determined and correlated to the NMR analyses, showing that the chemical structures of the released vancomycin and linezolid were maintained. It can be concluded that the manufacture of granules by wet granulation and isostatic compression, as well as the tests of release, changes neither the structure nor the activity of the vancomycin or linezolid, and thus, the antibiotics might be able to treat bone infection. However, a comparison of the amounts of activity detected by spectrophotometry and microbiological assay shows that only 28% of the vancomycin released from biomaterials prepared by dynamic compaction was active. This compaction technique is known to cause large but brief local temperature increases in compact materials, which are not quantifiable but high enough to induce grain joint formation. This process could denature vancomycin activity, which remains stable for 6 h at 80°C. As dynamic compaction reduces the microbiological activity of vancomycin, wet granulation and isostatic compression processes are preferred.

As a result, according to the nature of the implant and the release profiles of the different therapeutic agents, surgeons will be able to choose the most appropriate biomaterial (blocks, grains, or powders) for their patients.

4. CDA as a vancomycin delivery system: results

4.1 Rationale
In this study, we evaluated whether CDA could be used as a local DDS for vancomycin. The antibacterial activities of CDA loaded with 10% vancomycin over 14 days, in the presence or absence of a standard systemic treatment of vancomycin, were assessed using an in vivo model of acute MRSA osteomyelitis (Amador et al., 2010; Gaudin et al., 2011).

4.2 Materials and methods
4.2.1 Animals
Female New Zealand rabbits (2.0 to 2.5 kg) used in this study were individually caged and had free access to water and food. Experiments were performed according to the Committee of Animal Ethics of the University of Nantes, France. Animals soon to be moribund (i.e., having difficulty accessing water and food associated with 10% weight loss per day for 2 days) were euthanatized by lethal injection of thiopental under general anesthesia. A fentanyl patch (Durogesic®, Janssen-Cilag Lab.) was used for pain management. Due to the delay of action (approximately 12 hours), the patch was placed on the animals the night before beginning the experiment (induction) and changed every 72 hours.

4.2.2 Bacterial strain
The MRSA strain used in this study was isolated from a blood culture and exhibited heterogeneous, low-level methicillin resistance (methicillin MIC=16 μg/mL). Molecular characterization showed this strain has a cassette chromosome *SCCmec* type IVa and agr-1 and was Panton-Valentine leukocidin toxin- and toxic shock syndrome toxin-negative. The MRSA vancomycin MIC was 2 μg/mL. Inocula (CFU per mL) were adjusted to 10^9 CFU/mL.

4.2.3 Bacterial counts

Bacterial counts were determined after 48 hours of incubation at 37°C on tryptic soy agar plates. To evaluate whether vancomycin treatment induced the selection of in vivo-resistant variants, undiluted sample homogenates were spread on agar plates containing 8 µg/mL vancomycin, 4-fold greater than the MIC.

4.2.4 DDS synthesis

The most efficient concentrations of antibiotics were previously determined by in vitro and in vivo analyses (Amador et al., 2010). Granules containing 10% vancomycin were prepared by wet granulation. The size of the granules was determined to be 200-500 µm, compatible with common handling human practice.

4.2.5 Vancomycin tissue measurements

Dosages of vancomycin in joint fluid, bone marrow, and spongy bone were realized at $\lambda=214$ nm by HPLC (ThermoFisher Spectra System SCM1000/P1000XR, with automatic syringe AS3000 and UV6000LP detectors and FL3000). The column was a C18RP (Hypersil GOLD, 150-mm length, 5 µm × 4.6 mm, ThermoFisher). The isocratic mobile phase was 12% acetonitrile in an ammonium acetate buffer adjusted to pH 6 by phosphoric acid. Experiments with every range and dosage were performed in triplicate.

4.2.6 Experimental design

Animals were randomly assigned to six groups: (1) vancomycin group $V_{(IV)}$ [vancomycin constant intravenous infusion to reach a 20× MIC serum steady-state concentration (CIV)] for 4 days; (2) $V_{(CDA10\%)}$ (CDA loaded with 100 µg/mg vancomycin) for 4 days or (3) for 14 days; (4) $V_{(IV)}$ for 4 days + $V_{(CDA10\%)}$ (CDA loaded with 100 µg/mg) for 4 days (5) or 14 days (6) for vancomycin tissue measurement.

We used a percutaneous, transarticular route to perform femoral trepanation using a Jamshidi bone marrow biopsy needle under general anesthesia. The Jamshidi needle was inserted between the two femoral condyles and through the epiphysis, physis, and metaphysis to reach the medullar canal. After the needle was removed, the skin incision was closed. Subsequently a 1-mL suspension containing 10^9 CFU MRSA was injected parapatellarly into the knee cavity. At day 3, osteomyelitis was induced, causing unbridling, and the infected site was washed as recommended in human practice. Samples of joint fluid, bone marrow, and spongy bone were removed for bacterial counts. At day 7 or 17 post-inoculation, animals were euthanized to measure the bacterial load in the joint fluid, bone marrow, and bone. Results were expressed as the bacterial counts ($\log_{10}/g$ of tissue) at day 3 (reference level) and either day 7 or 17. The lower limit of detection for this method was 1 CFU/50 µl undiluted tissue homogenate. Infusions of antibiotics began at day 3 and continued for only 4 days due to ethical reasons.

4.2.7 Statistical analysis

Statistical analyses were performed using Graphpad Prism® 4 for Windows (Graphpad Software, San Diego, CA, USA). Results were expressed as the means ± standard deviation. Regimens were compared using one-way analysis of variance. This analysis was performed using a post-hoc Student-Newman-Keuls test. Time-dependant efficacy was tested by a non-

parametric t test associated with a Wilcoxon post-test. P less than 0.05 was considered statistically significant.

4.3 Results and discussion
4.3.1 Vancomycin tissue measurements

Wysocki et al. (2001) demonstrate that CIV patients (targeted plateau drug serum concentrations of 20 to 25 mg/L) reached the targeted concentrations faster, and fewer samples were required for treatment monitoring than with intermittent infusion patients (IIV). For comparable efficacy and tolerance, CIV may be a cost-effective alternative to IIV. Samples of plasma, spongy bone, and bone marrow were analyzed for vancomycin by HPLC in healthy and infected femurs. Vancomycin concentrations were 7.50 ± 2.48 µg/g in bone marrow and 6.11 ± 3.18 µg/g in spongy bone in healthy tissues, associated with a vancomycin plasma level of 19.09 ± 3.79 µg/mL. For infected bone tissues, vancomycin concentrations were 6.70 ± 2.23 µg/g, 12.63 ± 5.16, and 22.63 ± 8.53 for bone marrow, spongy bone, and plasma, respectively, related to the vancomycin MCIs of the causative MRSA (2 µg/mL). The constant infusion of vancomycin exhibits more than three times the MICs of the studied strain in any compartment.

For the CDA loaded with 10% vancomycin group (without a systemic approach), samples were taken at the maximum local delivering time (24 hours): plasma for vancomycin dosage and bone marrow and spongy bone, near the CDA implants, to assess local tissue delivery. Vancomycin plasma levels were 3.36 ± 0.81 µg/mL, corresponding to a very weak elevation. Local bone tissue concentrations were more than 100 times and 150 times the vancomycin MICs for the MRSA strain for bone marrow and spongy bone, respectively. Surprisingly, the introduction of CDA loaded with 10% vancomycin did not significantly increase the plasma concentration obtained at steady-state by continuous infusion of vancomycin, reducing the risk of general toxicity. In contrast, the contribution of local antibiotic-loaded material appeared to be considerable.

4.3.2 Bacterial counts

No vancomycin-resistant mutants were detected after either 4 or 14 days of treatment in any group and in any compartment.

Data from the in vivo experiments are summarized in Table 5. Because infusion caused major venous time-dependent impairment, the efficacy of $V_{(IV)}$ could not be assessed over the full 14-day treatment period. Moreover, $V_{(IV)}$ did not demonstrate significant antibacterial activity in any of the three tissues (joint fluid, bone marrow, and spongy bone) after the 4-day treatment. Of the different treatments [$V_{(CDA10\%)}$ and $V_{(CDA10\%)}$ + $V_{(IV)}$], only treatment with CDA-vancomycin plus constant infusion of vancomycin [$V_{(CDA10\%)}$ + $V_{(IV)}$] showed a significant inhibitory effect in joint fluid, with $P<0.05$ after 14 days of treatment.

Treatment with 10% vancomycin-loaded CDA alone exhibited a greater activity than $V_{(IV)}$ alone ($P<0.01$), but combining these treatments significantly enhanced treatment efficacy ($P <0.001$). $V_{(CDA10\%)}$ did not exhibit greater efficacy after 14 days compared with 4 days. In spongy bone and bone marrow, most samples were sterile after 14 days of treatment with CDA-vancomycin plus intravenous vancomycin [$V_{(CDA10\%)}$ + $V_{(IV)}$], but none after $V_{(CDA10\%)}$.

Furthermore, the combined $V_{(CDA10\%)}$ + $V_{(IV)}$ treatment seemed to be the most effective. These data indicate the addition of a local delivery system enhanced the antibacterial effects of these drugs in a tissue-specific manner. One possible mechanism for the enhanced efficacy of the combined treatment is that the release of vancomycin loaded onto CDA is limited to the site of infection, with cortical bone acting as a semi-permeable membrane and preventing the elimination of vancomycin through the bloodstream.

Treatment regimens	n	Means ±SD log10 CFU/g tissue								
		day 3			day 7			day 17		
		JF	BM	Bo	JF	BM	Bo	JF	BM	Bo
$V_{(IV)}$	10	7.88 0.62	6.97 1.15	7.86 0.44	7.78 ± 0.75	6.38 ± 1.89	7.29 ± 0.80	ND	ND	ND
$V_{(CDA10\%)}$	21	7.86 0.55	8.47 0.80	8.11 0.05	7.81± 1.03	5.06 ± 1.43	5.76 ± 1.45	6.27 ± 2	4.80 ± 1.56 [a]	6.16 ± 1.36 [a]
$V_{(CDA10\%)}$ + $V_{(IV)}$	15	7.53 0.69	8.58 1.32	8.41 1.36	6.63 ± 0.39	4.55 ± 1.33 [a]	3.78 ± 1.28 [a]	2.52 ± 0.97* [a,b]	2.33 ± 0.34 * [a,b]	2.30 ± 0.09 * [a,b]

ND: not done n: number of animals [a] *P<0.05 vs* day 3 [b] *P<0.05 vs* $V_{(CDA10\%)}$ and $V_{(IV)}$
*under the lower limit of detection

Table 5. Bacterial counts in joint fluid (JF), bone marrow (BM), and spongy bone (Bo) at day 3 (post-inoculation) and 4 and 14 days after the beginning of the treatment.

5. Conclusion

Biomaterials as local DDS could reduce or eliminate the toxic side effects and complications of systemic antibiotic treatments, enhancing patient safety. From this perspective, the reference antibiotic against MRSA, vancomycin, was selected, and optimized concentrations were loaded into a CaP matrix (CDA) by wet granulation. The in vitro antibacterial activity of eluents from the DDS showed that the nature of the antibiotics was not altered either as a result of CDA loading or after sustained release from the granules. After a 14-day in vitro release, the CDA matrix was still able to deliver antibiotics at a concentration 50 times greater than the MIC of the MRSA strain used, providing local effective bactericidal concentrations of antibiotic and not inducing the development of antibiotic resistance from a slow residual release at suboptimal antibiotic concentrations. While more traditional antibiotic carrier systems are available, CDA has both desirable antibiotic release kinetics and a high osteogenic-promoting activity, including degradation of the apatite, obviating the need for a second surgery to remove the implanted material.

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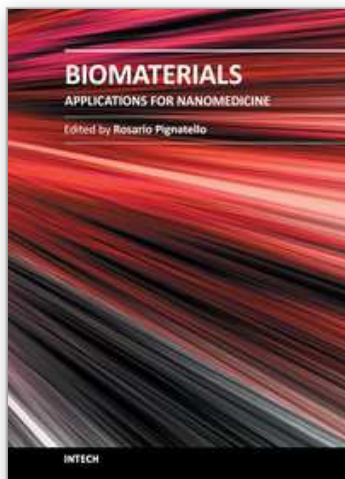
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