

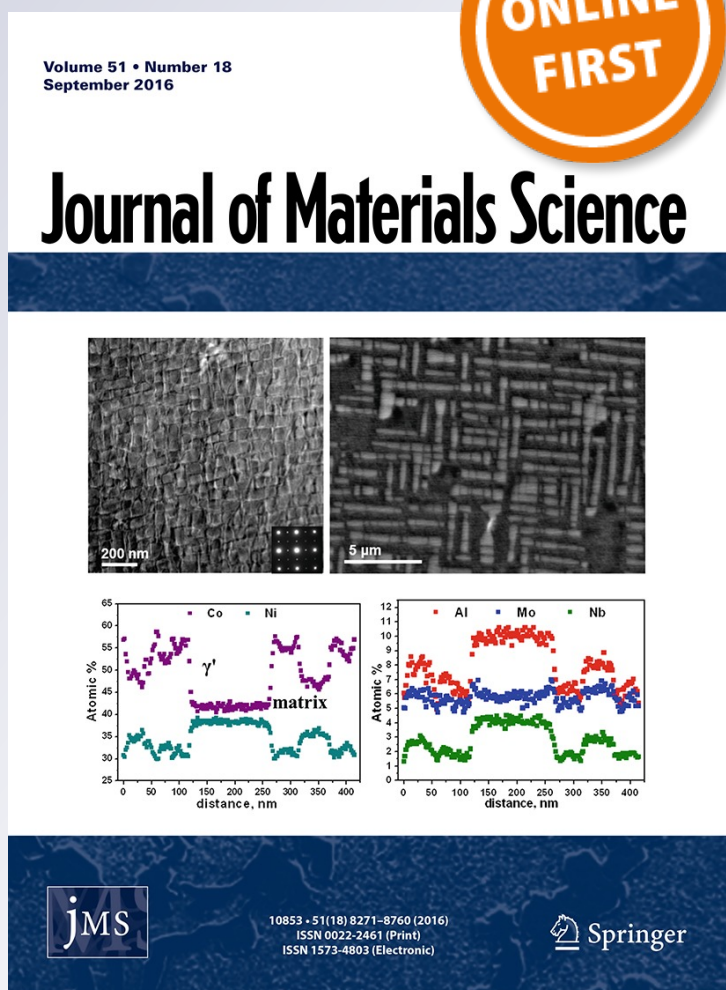
New colloidal fabrication of bioceramics with controlled porosity for delivery of antibiotics

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New colloidal fabrication of bioceramics with controlled porosity for delivery of antibiotics

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ABSTRACT

Bone tissue regeneration with bioceramics-based biomaterial can suffer from associated bone infections. The objective of this study was to develop new antibiotics drug delivery systems, composed of ceramics matrix with a controlled porosity aiming at releasing the antibiotics loaded in the matrix, in the bone implantation site and in a controlled way. Synthesis of the ceramics matrix is based on a colloidal approach. First, the heterocoagulation of hydroxyapatite nanoparticles (HA) and polymethylmethacrylate microspheres (PMMA) provides the control of the porosity in HA matrix. With constant size of microspheres and HA/PMMA ratio, the sintering temperature allows the modulation of interconnections porosity and volume. In this study, two sintering temperatures, resulting in two different porosities, are used. Secondly, porous biomaterials are loaded with tetracycline hydrochloride (antibiotics with large spectrum used in treatment of bone infections) by impregnation under vacuum. Control of the porous microstructure allows variation of the loaded quantity of antibiotics and of their release kinetics. The antibacterial properties of the ceramics loaded with antibiotics vary with the porous microstructure, both in terms of bacterial growth in the ceramic surroundings and of bacterial colonization of the ceramic surface. Protein adsorption from surroundings onto the ceramic surface varies depending on the porous structure, however without inducing significant changes of antibacterial properties. Unloaded and loaded porous bioceramics are biocompatible with pre-osteoblastic cells as shown by *in vitro* evaluation. The investigation of DDS with two different porosities demonstrated the possibility to control the antibiotics loaded quantity and the antibacterial efficiency by varying the matrix porosity. Release kinetics is also affected by changes in ceramic porosity but in a much more limited way.

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Introduction

Infections on biomaterials are a common cause of implantation failure often forcing the removal of the infected material [1]. Biomaterial-associated infections are due to bacterial biofilms developed on the implant surface after initial contamination occurring during surgery or due to bacteria coming from infections in the patient body (e.g. dental infections). Mostly, systemic antibiotic treatment is ineffective since biofilms provide protection for bacterial cells through restrictions in molecule diffusion and bacteria sensitivity [2]. Then, infections become chronic. The development of new antibacterial and anti-biofilm strategies is needed for fighting locally against biofilm growth. In this framework, bioactive biomaterials able (i) to release antimicrobial drugs with a predictable kinetics, (ii) to deliver drug in a specific location with a controlled local drug concentration and (iii) to limit adverse systemic effects by allowing reduction of the delivered doses of drug, are of major interest.

For bone tissue regeneration, hydroxyapatite (HA) is a common and well-known biomaterial. Hydroxyapatite-based ceramics materials providing antibacterial effect through antibiotic release have been proposed for designing implantable drug delivery system (DDS) [3–8]. In these bioactive materials, selection of the most suitable ceramic matrix is crucial but difficult. In particular, pore size and structure of the inorganic host system are key factors for controlling both quantity and release kinetics of the drug [9–17]. Various techniques exist for the fabrication of porous ceramics. They can be classified into 2 categories. The first one is an additive manufacturing technique based on layer-by-layer building of 3D scaffolds from a computer-aided design file [18–20]. The second approach, based on a colloidal process, is more conventional and consists in the generation of porosity inside the ceramic through various routes [21, 22]. One possibility is the impregnation of a cellular template (e.g. polyurethane foam) with ceramic suspension, before elimination of the template by a thermal treatment, providing the positive replica of the template [23]. Another method is based on the incorporation of a porogen agent that is finally eliminated by thermal treatment or dissolution [24–26], resulting in the porous ceramic. In a third method (direct foaming),

porous materials are produced by incorporating air into the ceramic suspension [27, 28]. In general, versatility of these colloidal methods for the control of porosity is low, leading to pores with irregular size and spatial distribution. Here, we focus on a process using heterocoagulation, which allows the control of pore size, pore distribution in the massive material and volume of porosity [29, 30]. Micropores are expected to be obtained thanks to the presence of polymethylmethacrylate (PMMA) microspheres in a suspension of HA nanoparticles. Pore size is controlled by the size of the microspheres, while homogeneity of pore distribution results from the dispersion of the initial HA/PMMA suspension. Porosity can be varied by adapting the volumic ratio between HA particles and PMMA spheres [31].

Our first objectives were the synthesis of HA-based ceramics with a controlled porosity, and the control of loading and *in vitro* release of antibiotics thanks to control of the porous microstructure. Two different microstructures were chosen for their good compromise between interconnection rate and matrix cohesion/stability allowing to expect satisfying mechanical properties [32]. They were loaded by *in vacuo* impregnation [16] with tetracycline hydrochloride (TC), a large spectrum antibiotics used for bone infection treatments [33]. Our second objective was to investigate the antibacterial properties of these new DDSs and to confirm their biocompatibility for mammalian cells.

Here, HA matrices with two different microstructures will be described, especially in terms of porosity (pore rate, pore size distribution, and interconnections). Their properties as DDS will then be investigated based on their ability to adsorb and release TC. In a second part, the antibacterial properties of the DDS will be reported. Short- and middle-term antibacterial effects for both bacteria in the near surrounding of the DDS and bacteria adherent to the material will be addressed in particular. Additionally, *in vitro* biocompatibility of the developed DDS was evaluated on eukaryotic cells. Since implantable biomaterials are usually suited in complex surroundings such as body fluids, they are subject to the adsorption of a large range of biochemical components, including proteins for a large part, which may rapidly condition their surface before mammalian cells and bacteria adhere [34]. Adhesion of mammalian cells and bacteria may thus be affected by changes in chemical properties of the

material surface. In addition, adsorbed components of the conditioning film may affect delivery properties of the DDS by forming a barrier and preventing bioactive agent release. In this frame, biomolecules in cell culture medium are expected to adsorb onto HA-1100 and HA-1220 DDS. The potential barrier effect of proteins previously adsorbed onto the HA surface has been therefore investigated and will be here briefly discussed.

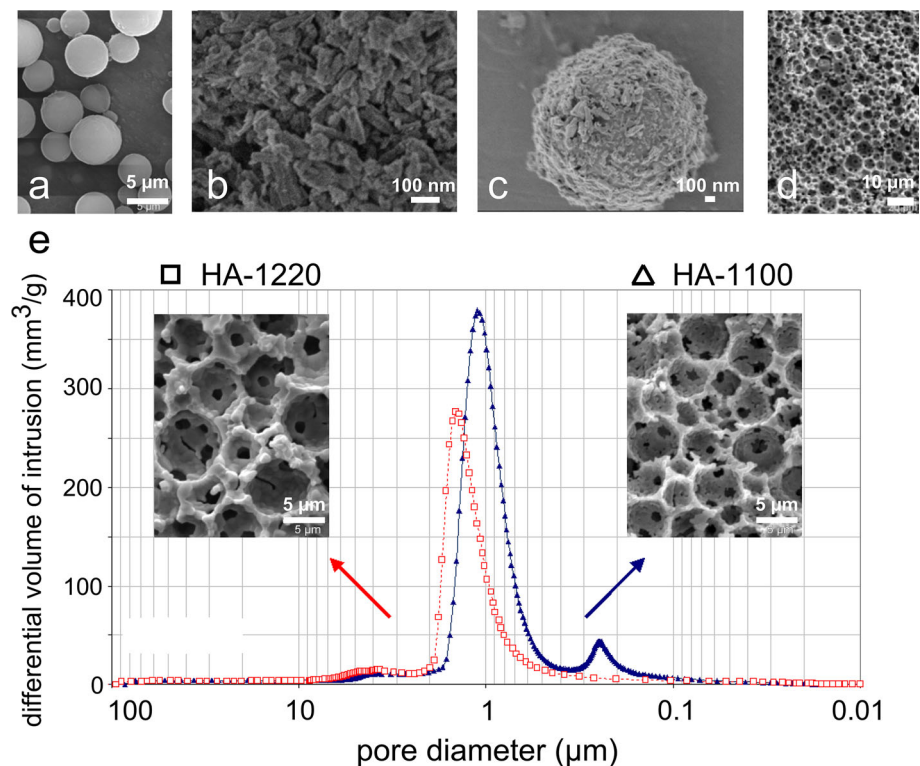
Materials and methods

Preparation and characterization of porous materials (HA DDS): heterocoagulation process

Preparation and characterization of the porous materials are described elsewhere [29]. Briefly, microspheres of polymethylmethacrylate (PMMA) were used as a structuring agent (Fig. 1a). Mean diameter of spheres was 7 μm and zeta potential was -30 mV at pH 6.6 in aqueous medium. Powder of stoichiometric hydroxyapatite (HA, Fig. 1b) was synthesized by co-precipitation in aqueous medium, as previously described [35]. It was then re-

suspended in aqueous medium, dispersed and stabilized by addition of polycationic dispersant. Mean size of hydroxyapatite particles dispersed in suspension was 0.1 μm and zeta potential was $+50$ mV between pH 6 and 7. Suspensions of HA and PMMA were mixed at a HA/PMMA ratio of 0.42 as previously determined [31]. The resulting suspension was filtered to remove solvent. Pressure created by pumping caused the compaction of HA/PMMA particles, leading to the formation of thin cylinders (~ 12 mm in diameter and 1–2 mm in thickness) of an organic/inorganic composite. Drying of the composite material was achieved in oven before thermal treatment at 500 $^{\circ}\text{C}$ aiming at the removal of the PMMA microspheres. The material was finally sintered at 1100 or 1220 $^{\circ}\text{C}$ to provide final ceramics with two different porosities (materials named HA-1100 and HA-1220, respectively). The resulting material samples were thin cylinders of 11.4 ± 0.5 mm in diameter and 1.2 ± 0.3 mm in thickness. The porous microstructure of the final materials was confirmed by Scanning Electronic Microscopy (Hitachi S2500). Pores and interconnections characteristics (rate and size) were determined using mercury porosimetry (Micromeritics Autopore 9510). Three different HA samples were used

Figure 1 a PMMA microspheres, b hydroxyapatite (HA) nanoparticles, c PMMA microspheres coated by HA nanoparticles, d porous ceramics after sintering, e porogram and SEM micrographs of porous ceramics synthesized with a 0.42 HA/PMMA volume ratio and sintered at 1100 $^{\circ}\text{C}$ (HA-1100) and 1220 $^{\circ}\text{C}$ (HA-1220).



together to provide one measurement and measurement was carried out once. Porosity was also calculated on the basis of weight, area, and thickness of the HA samples according to Eq. 1, where HA density is 3.065 g/cm^3 . This porosity was determined for each sample.

$$\text{Porosity}(\%) = \left(1 - \frac{\text{Weight}}{\text{Area} \times \text{Thickness} \times \text{HA density}}\right) \times 100 \quad (1)$$

Preparation and characterization of porous materials loaded with tetracycline (TC-HA DDS)

Tetracycline hydrochloride (TC) (Supporting Information, Fig. 1a; MIC₅₀ of 1–16 $\mu\text{g/mL}$ for *Escherichia coli* (*E. coli*) [36]) was purchased by Cooper (Melun, France) as a yellow powder. Porous ceramics were impregnated under vacuum conditions with solutions of TC in ethanol during 30 min, before drying in air at 37 °C. Concentrations of TC in ethanol ranged from 5 to 15 mg/mL. For each of these initial concentrations, the quantity of TC loaded in the porous ceramics was determined by using UV-spectrophotometry (Lambda 25, Perkin Elmer) at 276 nm after achievement of total dissolution of TC-HA samples in acid solution (HCl, 2.4 M). For the concentration finally used for biological assays (5 mg/mL of tetracycline in ethanol), measurement of TC quantity in porous ceramics was also achieved by Temperature Programmed Desorption, as described in Gadiou et al. [37]. Tetracycline degradation fragments with $m/z = 50$, that may be the result of various pathways of the complex degradation of TC [38–41], were used as a signature of the tetracycline molecules loaded in HA matrix. A calibration curve was provided in a range of TC quantity from 0 to 2.5 mg (Supporting Information, Fig. 1b). Results are presented as the mean value \pm standard deviation of at least three replicates for the first method, two replicates for the second method.

In order to investigate the incidence of the porous structure of the HA matrix, the property of release of the DDS was assayed on TC-HA-1220 and TC-HA-1100 containing the same quantity of adsorbed TC molecules. For that purpose, the kinetics of TC release were determined in aqueous medium and under controlled conditions (50 mL of water,

continuous stirring, pH 7, $T = 37 \text{ }^\circ\text{C}$). Assaying by using UV-spectrophotometry (Lambda 25, Perkin Elmer) at 276 nm provided the concentration of dissolved tetracycline. Kinetics curves of TC release from porous materials are finally expressed as percent of released TC compared to the total mass of TC loaded in the material, determined by total dissolution as described above.

All incubation steps of biological and microbiological assays were performed in the dark to avoid any photo-degradation of tetracycline molecules.

Bacterial species and growth conditions

Before microbial characterization, samples were sterilized by UV of 254 nm wavelength during 7 min at 2 cm from UV-C lamp.

Gram-negative *E. coli* were chosen as a model for pathogenic bacteria species among the most frequently implicated in implant-associated infection [42–44]. Experiments were conducted with *E. coli* PHL628 (*E. coli* MG1655) known to produce curli and exocellular polymeric substances and to attach to abiotic surfaces [45]. After defrosting of 1 mL of bacteria stored at $-80 \text{ }^\circ\text{C}$, three successive pre-cultures were grown at 30 °C (10 % vol. of the previous pre-culture in fresh medium). Both first steps of pre-culture were grown overnight whilst the last one was grown for 4 h and was used to inoculate the final culture (10 % vol. of the last pre-culture). All bacterial cultures were done in a selective medium (M63G [45], pH 6.8) to avoid any uncontrolled adsorption of biomolecules coming from the medium. Concentrations of bacteria in aqueous suspensions were determined by absorbance measurements at 600 nm ($\text{Abs}_{600\text{nm}}$) under UV-visible spectrometer. $\text{Abs}_{600\text{nm}}$ were then converted in number of bacterial per volume by using adequate calibration relationships.

Adhesion inhibition of bacteria onto DDS and growth inhibition of adhered bacteria

The ability of TC-HA DDS to inhibit in vitro bacterial adhesion and/or growth of bacteria adhered on DDS surface was evaluated by measuring the adhered bacterial population and estimating their cultivability. Sterilized samples were inoculated with 4 mL of M63G medium containing 5×10^6 bacteria/mL ($\text{Abs}_{600\text{nm}}$ initial of 0.01) and incubated for 2 h at 30 °C. Then, samples were rinsed three times with 2 mL of

fresh NaCl solution (9 g/L in water) to collect non-adherent bacteria without creating any air-material interface [46]. Abs_{600nm} of initial supernatants (i.e. supernatants after 2 h of culture) and of rinsing solutions (Supporting Information, Fig. 2) were measured and were summed for each sample to assess the amount of planktonic bacteria after culture. Corresponding inhibition rates were measured by $(Abs_{600nm\ initial} - Abs_{600nm}) / Abs_{600nm\ initial}$. These inhibition rates are called "Inh_{plank1}" in the following. Assays were repeated 5 times for each HA and TC-HA DDS. Results are given as the average of the replicates. After NaCl 9 g/L rinsing, HA and TC-HA DDS samples were used for the evaluation of quantity and cultivability of adhered bacteria.

Bacterial colonization on DDS samples was analysed in situ in the last NaCl 9 g/L rinsing solution by using a Confocal Laser Scanning Microscope (CLSM)

(Zeiss LSM700 mounted on an upright microscope and equipped for fluorescence and reflection microscopy modes) equipped with a water immersion objective (W Plan Apochromat $\times 63/1.0$) for keeping bacteria in satisfying physiological conditions. 3D images were performed with the reflection mode for allowing characterization of the material surface topography and with the fluorescence mode after Syto9[®] (Molecular Probes, 1 μ L of 5 mM Syto9[®] stock solution per mL of NaCl) fluorescent staining for visualizing adhered bacteria. Quantity of adhered bacteria was determined after treatment of the 3D images using ImageJ software [47] and image analysis using CellC software [48]. This experiment was realized in triplicate. Two samples of each HA and TC-HA DDS type were observed for each experiment. Between five and ten random locations ($102\ \mu\text{m} \times 102\ \mu\text{m}$) were imaged with CLSM on each

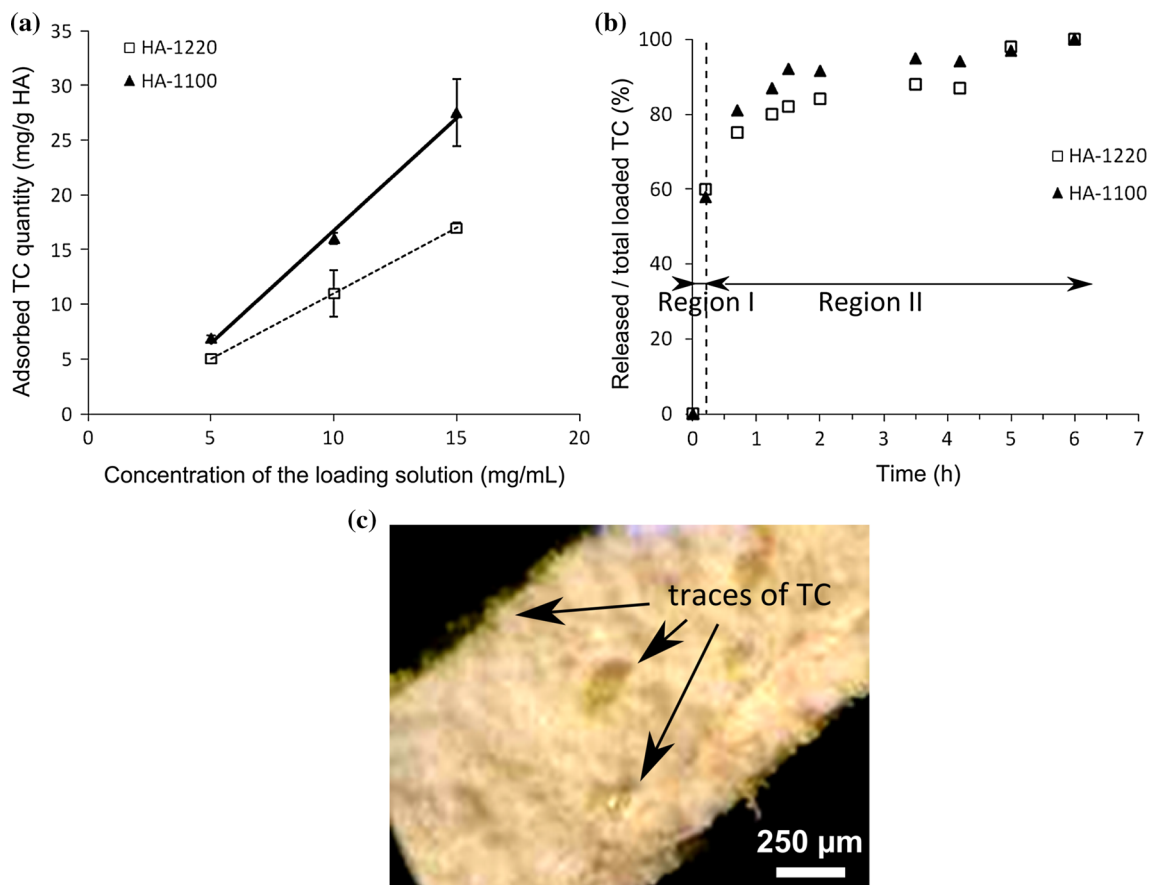


Figure 2 Behaviour of TC-HA DDS throughout TC loading and releasing. **a** Quantity of TC loaded in HA porous matrix as function of the matrix porosity and of the concentration in TC of the loading solution. **b** Kinetics of TC releasing from TC-HA DDS for 6 h in 50 mL of water, showing two different regimes of

release. **c** Optical micrograph (Optical microscope MZ16, Leica) of a fractured TC-HA sample before TC releasing process, showing the presence of TC on the surface and in the inner pores of the matrices (yellow traces pointed out by black arrows).

sample. Imaging of sample border was avoided. Results are given as the average of 20–40 values. Corresponding inhibition rates are called “Inh_{adh}” in the following.

The cultivability of adhered bacteria was estimated by a so-called “print test”. Sample top sides were pressed for 5 s on a LB-agar plate before removing. LB-agar plates were incubated at 30 °C for 16 h. The capacity of so-transferred bacteria to form colonies provided an indication of the cultivability of adherent bacteria. This experiment was repeated twice for each HA and TC-HA DDS type.

Growth inhibition of bacteria in DDS surroundings

Antibacterial effects of TC released from DDS on bacteria present in surroundings were evaluated both in semi-solid and in liquid media, aiming at the characterization of the TC-HA DDS ability to inhibit the growth of bacteria living in the material surroundings.

The antibacterial effect on semi-solid surroundings was evaluated by measuring areas of bacterial growth inhibition around samples loaded with antibacterial agent and placed on previously inoculated agar plates (modified Agar Diffusion Method [49]). Briefly, 100 µL of fresh bacterial suspension was spread on lysogeny broth (LB) agar-supplemented (15 g/L) growth medium to form a thin film of bacterial suspension. Sterilized HA DDS and TC-HA DDS samples were placed in contact with the previously homogeneously inoculated agar plate (top side of samples in contact with agar). After 24-h incubation at 30 °C, diameters of zones without growing of bacteria (so-called Inhibition Zones, visible around TC-HA DDS samples) and diameters of the samples were measured to allow further determination of the inhibition areas. Experiment was run three times for each HA type (one HA DDS sample and the corresponding TC-HA DDS sample per agar plate). Results are given as the average of the three replicates.

Aside from the measurements performed in the supernatants of samples used for adhesion experiments (Inh_{plank1}, see section above), the antibacterial effect in liquid surroundings was also evaluated by determining the inhibition rates of growth of planktonic bacteria in the presence of DDS in culture medium for durations up to 7 days. Sterilized

samples were placed in 22 mL of M63G medium containing 5×10^6 CFU/mL bacteria (Absorbance at 600 nm – Abs_{600nm initial} of 0.01) and incubated for various periods (1, 2, 8, 24, 48, 72, 168 h) at 30 °C. After incubation, 1.5 mL of suspension was taken twice and re-injected after determination of bacterial concentration, which was performed by measuring absorbance at 600 nm (Abs_{600nm}). Inhibition rate corresponding to each sample was then calculated according to $(\text{Abs}_{600\text{nm initial}} - \text{Abs}_{600\text{nm}}) / \text{Abs}_{600\text{nm initial}}$. These inhibition rates are called “Inh_{plank2}” in the following. Experiment was run 5 times for each type of TC-HA DDS. Results are given as the average of the replicates.

Protein adsorption

Potential impact of protein adsorption on the TC-related antibacterial effect has been investigated. Samples of each HA and TC-HA DDS type were kept 1 h under hydrodynamic culture conditions in cell growth medium (McCoy's, Sigma-Aldrich) containing 10 % of foetal bovine serum (FBS) and 1 % L-glutamine but without any additional antibiotics for not disturbing the analysis. Samples were placed in perfusion bioreactors (Minucells and Minutissue, Germany). Medium flow through DDS samples was maintained at 2 mL/h rate [50]. Biochemical and physicochemical analyses were performed during and after dynamic experiments. Quantity of proteins in liquid medium before and after passage through DDS was measured using BCA assay (ThermoFisher Scientific). Quantity of tetracycline released from the TC-HA DDS during dynamic adsorption of proteins was estimated by measuring the absorbance value at 276 nm (Abs_{276nm}) before and after dynamic experiment. Surface morphology and chemical composition of HA and TC-HA DDS after dynamic experiment were investigated by scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS) analysis. SEM images were performed using a FEI Quanta 400 microscope after gold metallization. XPS survey spectra were recorded with a gamma SCIENTA SES 200-2 spectrometer equipped with a monochromatized KR1, 2 anode (1486.6 eV) and a concentric hemispherical analyzer. Photoemitted electrons were collected at a takeoff angle of 90° from the substrate, with electron detection in the constant analyzer energy mode. The survey spectrum signal was recorded with pass energy of 500 eV. Bacterial

growth inhibition assays were finally conducted with DDS treated with 1 h of dynamic experiment by following the same procedure as previously described (“Growth inhibition of bacteria in DDS surroundings” section) at 1, 2, 8 and 24 h of incubation time. These inhibition rates are called “ $\text{Inh}_{\text{plank with proteins}}$ ” in the following. All these analyses were done on three different samples of each HA and TC-HA DDS type except SEM and XPS analyses (one sample of each type for each characterization technique). Results are given as the average of the replicates.

Biocompatibility tests

Compatibility of TC-HA DDS with eukaryotic cells was tested for HA-1220 and TC-HA-1220 only, which were chosen for the better compactness and stability of pore walls. Experiments were conducted with MC3T3-E1 cells (immortalized pre-osteoblastic mouse cell line, kindly provided by Dr M. Hindie (Errmece laboratory, Cergy-Pontoise University, France)). All products were provided by Sigma-Aldrich in the absence of any indication to the contrary.

Biocompatibility of released compounds was tested by cell culture in HA-1220 and TC-HA-1220 extracts. According to a weight/volume ratio of 0.1 g/mL, material samples were immersed into incomplete ISCOVE's culture medium (i.e. supplemented with 1 % L-glutamine, 100 U/mL penicillin-G and 0.1 mg/mL streptomycin sulphate but without FBS). Immersion was maintained 5 days at 37 °C for allowing the total release of TC molecules from the DDS samples. The so-obtained extract solution was further used without (100 %) or with dilution in incomplete ISCOVE's medium (50 %) and supplementation with 10 % FBS. 100 and 50 % solutions were used to replace the culture medium of cells previously cultured for 24 h at 37 °C under 5 % CO₂ condition in complete ISCOVE's medium (incomplete ISCOVE's medium supplemented with 10 % FBS) in a 96-well culture plate seeded with 2×10^4 cells per well. After 24-h incubation at 37 °C, media were replaced by 100 μL of 0.5 mg/mL MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide [51]) before 4-h incubation at 37 °C and

under 5 % CO₂ condition. MTT media were then replaced by 100 μL of acidic isopropanol (300 μL HCl + 100 mL isopropanol) for 20-min incubation at 37 °C. Acidic isopropanol was detected by measurement of absorbance at 570 nm.

Reliability of HA-1220 and TC-HA-1220 DDS for the development of attached cells was investigated by direct contact culture on the material surface. 1 mL of cells in complete ISCOVE's medium (4×10^4 MC3T3-E1 cells/mL) was used to seed each material sample (3 samples of each material type). After 6, 24 and 72 h of incubation at 37 °C and under 5 % CO₂ condition, culture medium was replaced by 500 μL of PrestoBlue[®] solution (Molecular Probes[™]) and incubated for 1 h at 37 °C before absorbance measurement at 570 nm. The absence of toxicity of PrestoBlue[®] solution [52] allowed further analysis of cells by fluorescent staining and microscope observations. Cells were fixed with a solution of 2 % (v/v) paraformaldehyde in NaK₂P buffer for 20 min and permeabilized with 0.2 % (v/v) of Triton X-100 for 15 min. Non-specific staining was prevented by a 20-min treatment with 1 % solution of bovine serum albumin (BSA) in phosphate buffered saline. DNA in nuclei and actin filaments were stained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI, 100 ng/mL) and phalloidin-TRITC (0.4 mg/mL), respectively. Three locations (386 $\mu\text{m} \times 203 \mu\text{m}$) were observed per sample by using CLSM equipped with a water immersion objective (W Plan Apochromat $\times 63/1.0$). Number of nuclei per micrograph, average area and circularity of cells were analysed with ImageJ software [47].

Statistical analysis

All biological experiments were run at least 3 times. For all replicates, at least 2 material samples were analysed. Results are presented as average and standard deviation of all replicates. Significance of differences was tested by two-by-two bilateral Student's *t* tests (application conditions: independent data and equal variances assessed by *F* test) with significance thresholds (α) of 0.01 and 0.05. According to Scherrer [53], the alternative hypothesis (H_1 : $\mu_X \% \neq \mu_Y \%$) was assumed to be true when the main hypothesis (H_0 : $\mu_1 = \mu_2$) was rejected.

Results and discussion

Synthesis and characterization of HA DDS

HA/PMMA suspension was formed by mixing HA and PMMA dispersions. Heterocoagulation of both components was expected from electrostatic attraction between the oppositely charged nanometric HA particles and micrometric PMMA spheres. This was confirmed by observation of the mixture with SEM, which highlighted the formation of core/shell structures composed of PMMA microspheres covered by HA nanoparticles (Fig. 1c). After filtering to remove solvent, thermal treatments of the organic/inorganic composite were performed to remove PMMA particles and achieve sintering. Porous ceramics were obtained as final products. Figure 1d shows the typical porous structure of ceramics synthesized with a 0.42 HA/PMMA ratio and sintering temperature of 1220 °C (The micrograph was performed after fracture of a ceramic sample). Micropores are spread homogeneously in the material and are all interconnected. The spherical shape of PMMA spheres is preserved in the shape of the micropores.

Both porous ceramics used as HA matrices in the present work were obtained with shell-to-core ratio of 0.42. They differed in sintering temperature that was fixed at 1100 and 1220 °C for the so-called HA-1100 and HA-1220, respectively. As shown in Fig. 1e, characteristics of the matrix porosity vary accordingly to the sintering condition. The porogram also displays a low dispersion of pore and interconnection sizes for specific sintering condition. Porosity

volume, pore and interconnection sizes resulting from the porosimetry analysis are summarized in Table 1. Pore size distribution is bimodal and trimodal in HA-1220 and HA-1100, respectively, and two populations of pores are shared by both HA matrices. On the basis of SEM micrographs, the population of pores with diameter from 2.3 to 10 μm can be attributed to the elimination of the porous agent i.e. PMMA microspheres. The second population with pore diameter in the [0.4–2.3 μm] range is attributed to interconnections with mean diameter of 1.1 μm for HA-1100 and 1.4 μm for HA-1220. The third population of pores was only highlighted in HA-1100 in a diameter range of [0.1–0.4 μm]. These pores only observed on SEM micrographs of HA-1100 and with a mean size of 0.2 μm were attributed to interconnections resulting from lower sintering temperature as compared to HA-1220. Additionally, porosity (72.5 %) and volume of interconnections (698 mm^3/g) were higher in HA-1100 than in HA-1220 (59.1 % and 438 mm^3/g), leading to less compactness of the pore walls in HA-1100 compared to HA-1220 (Fig. 1e). Sintering temperature was thus demonstrated to modulate the porosity and the size and volume of interconnections without changing size of PMMA microsphere and HA/PMMA ratio. Besides, measurement of HA material densification and subsequent calculation of the corresponding porosity of each sample used in the present work (mean values and standard deviations reported in Table 1) depicted a good reproducibility of the fabrication process, with a low relative variation of the porosity throughout the whole sample population

Table 1 Porosity characteristics of porous HA matrices (HA-1100 and HA-1220) obtained after sintering at temperature of 1100 and 1220 °C, respectively

	HA-1100	HA-1220
Calculated porosity ^a (%)	73.3 ± 1.3	65.7 ± 2.5
Measured porosity ^b (%)	72.5	59.1
Mean interconnection pore size (μm) in diameter range of [0.1–0.4 μm]	0.23	Multidisperse distribution (see Fig. 1)
Mean interconnection pore size (μm) in diameter range of [0.4–2.3 μm]	1.1	1.4
Mean porous volume (mm^3/g) in diameter range of [0.1–0.4 μm]	57	4
Mean porous volume (mm^3/g) in diameter range of [0.4–2.3 μm]	698	438

^a Porosity was calculated on the basis of densification

^b Porosity was measured by mercury porosimetry

compared to the mean value (1.8 and 3.8 % for HA-1100 and HA-1220, respectively).

TC-HA materials

TC quantities loaded in HA matrices for various concentrations of the loading TC solutions were determined by total dissolution of HA in acid solution (Fig. 2a). As expected, variation in loaded TC quantity was observed to follow HA matrix porosity: Matrix presenting higher porosity volume and more interconnections, as highlighted for TC-HA-1100, allowed the loading of higher quantities of TC compared to HA matrix with a more compact structure (TC-HA-1220). This is attributed to the higher surface of adsorption offered by the porosity and the interconnections of TC-HA-1100 in agreement with theoretical and experimental results reported in the literature [9, 16]. In addition, the loaded TC quantity increases linearly with TC concentration of the loading solution for both HA matrix types. Further antibacterial assays were conducted with TC-HA-1100 and TC-HA-1220 DDS of the lowest TC-loaded doses i.e. obtained by loading 5 mg/mL TC solutions in ethanol. Their corresponding doses of adsorbed TC, as determined by total TC-HA dissolution in acid and by TPD measurement, are reported in Table 2. Results of both methods are in very good accordance and confirm the higher quantity of TC loaded in TC-HA-1100 compared to TC-HA-1220.

The release properties of both TC-HA DDS types were compared for equal doses of adsorbed TC. Thus, kinetics of TC release were analysed for TC-HA-1100 and TC-HA-1220 samples loaded with 15 mg of TC per g of HA, obtained with impregnation with a TC solution of 10 and 15 mg/mL, respectively (Fig. 2a). Assays were conducted for 6 h in 50 mL of water (Fig. 2b). As determined before and reported in Table 2, loaded TC quantities were 0.44 ± 0.05 and 0.32 ± 0.07 mg for TC-HA-1100 and TC-HA-1220, respectively. Considering the total release of TC, maximal concentrations were equal to 0.008 and 0.006 mg/mL, broadly lower than the

solubility of TC (33 mg/mL in water and for temperature of 15–25 °C). Therefore, SINK conditions were respected (medium volume $\gg 3 \times$ volume at solubility limit) and the full dose of tetracycline was consequently allowed to be released. The structure of HA matrix was maintained throughout the release assays, demonstrating the stability of the DDS system (data not shown). For all the tested samples, 100 % of the total loaded quantity of TC was released throughout the assays, showing that the total release was achieved (Fig. 2b). In addition, two different regimes were distinguished in the kinetic curves, with a first, fast release of TC from DDS, up to 60 % of the total loaded quantity of TC (Region I in Fig. 2b), and, in a second time, a significantly slower release from DDS of TC molecules still entrapped in DDS ceramic matrix (Region II in Fig. 2b). The difference in release speed is attributed to the origin of the released TC molecules: Fast release, observed in region I and similar for the two samples, might be related to TC molecules adsorbed on the surface of pores directly open to the outer sides of the DDS sample, while slower release might result from TC molecules adsorbed in the porous HA material core. Such presence of TC in the internal pores is supported by optical micrographs of fractured DDS samples (Fig. 2c) that clearly show the capability of TC loading under vacuum conditions to drive TC molecules (here visible as yellow TC crystals) into the core of the DDS samples. Furthermore, the second region of the kinetics curve attributed to the porous core slightly but significantly differs between TC-HA-1100 and TC-HA-1220. $T_{80\%1220}$ (time necessary to release 80 % of TC loaded on HA-1220) is almost two times higher than $T_{80\%1100}$ (time necessary to release 80 % of TC loaded on HA-1100) (1.25 and 0.70 h, respectively), showing a faster release from TC-HA-1100 in agreement with more interconnections able to favour release of TC adsorbed in the material core. In comparison with other works on DDS such as Murugan et al. [54] and Domingues et al. [55], the release of TC appeared to be relatively fast. This can be explained by the experimental conditions that are

Table 2 Quantity of TC molecules loaded in HA matrix and determined both by TPD and by total releasing

	Value measured by TPD (in mg per g of HA)	Value measured by total dissolution (in mg per g of HA)	Value measured by total dissolution (in mg per sample)
TC-HA-1100	5.8 ± 1.6	6.9 ± 0.3	0.44 ± 0.05
TC-HA-1220	4.8 ± 0.1	5.0 ± 0.4	0.32 ± 0.07

known to directly impact release kinetics [56]. Indeed our priority was to study the effect of porosity on the kinetics using standardized experimental conditions and not to predict the *in vivo* release. Therefore, the choice was made to perform the trials in 50 mL of dissolution medium under agitation and to take aliquots for the TC dosage as a function of time. These conditions limit variations all along the duration of the release, but they can lead to a faster release of the drug substance in comparison with trials performed using dissolution medium volumes of a few mL. In addition, considering the notable difference in diffusion offered by biological tissues and biofilms compared to aqueous liquids [57–60], release kinetics of TC is expected to significantly slow down in *in vivo* conditions compared to *in vitro* ones [61, 62]. *In vivo* duration for total release should therefore finally continue far beyond the *in vitro* determined 6-h duration.

Bacterial growth inhibition

The efficiency of TC-HA DDS for preventing development of bacteria in its near surroundings i.e. resulting from TC molecules released and diffused in the liquid or semi-solid medium, was evaluated for planktonic bacteria living in liquid culture medium and for bacteria growing on semi-solid nutritive agar plates.

Significant inhibition of bacterial growth was observed in the presence of TC-HA DDS in both liquid and semi-solid culture media (Fig. 3), clearly highlighting that the TC efficiency was conserved after the loading and releasing processes. Inhibition areas were observed on the nutritive agar plates used for the diffusion assay, around both the TC-HA-1100 and the TC-HA-1220 samples placed top-down on the inoculated agar plates (Fig. 3a). The inhibition area was slightly higher with TC-HA-1100 ($9.1 \pm 1.3 \text{ cm}^2$) compared to TC-HA-1220 ($8.4 \pm 0.7 \text{ cm}^2$) in agreement with the higher amount of TC loaded in TC-HA-1100 (Table 2), but differences were not significant. The inhibition efficiency measured in liquid culture medium was determined by comparing the concentration of planktonic bacteria in 4 mL of culture medium in the presence of TC-HA (i.e. with TC concentration of 110 ± 12 and $80 \pm 17 \text{ } \mu\text{g/mL}$, see Table 2) and HA samples for 2 h. Results shown in Fig. 3b display a reduction of more than 50 % of planktonic population growth in the surroundings of

HA-TC DDS compared to HA DDS. Inhibition of bacteria growth was not significantly different between materials obtained with different sintering temperatures ($\text{Inh}_{\text{plank1}} = 51 \pm 8 \%$ for TC-HA-1100; $\text{Inh}_{\text{plank1}} = 53 \pm 1 \%$ for TC-HA-1220). In addition, inhibition rates were in accordance with the expected values ($45 \pm 5 \%$ for 100 $\mu\text{g/mL}$ of TC with pre-culture and culture in M63G; see Supporting Information, Fig. 3), knowing the maximal concentration of TC reached in 4 mL of M63G medium with one TC-HA DDS sample (110 and 80 $\mu\text{g/mL}$ for TC-HA-1100 and TC-HA-1220 respectively). It should be noted that susceptibility of bacteria to antibacterial agent is strongly dependent on the growth conditions, especially on the composition of growth medium [63]. As we experimentally confirmed, inhibition rates in M63G medium are lower than in LB culture medium, commonly used in literature (about 80 % in LB medium supplemented with 100 $\mu\text{g/mL}$ of TC) (Supporting Information, Fig. 3). Higher efficiency of TC-HA DDS can be therefore expected under *in vivo* conditions of use.

Kinetics of planktonic bacteria growth in the presence of TC-HA DDS was analysed for 7 days in 22 mL of liquid nutritive medium (Fig. 3c, d). For both DDS types, resulting inhibition rate displayed two regimes: inhibition rate increased rapidly up to about 60 % during the first 8 h, while it only slowly rises during the next 6 days, reaching about 85 % after 7 days of culture in the presence of TC-HA DDS (Fig. 3c). The rapid inhibition increase can be attributed to the high doses of TC released in culture medium during the first period (as reported above, in 50 mL of water, about 100 % of the loaded TC quantity in the 6 first hours) i.e. 0.020 and 0.015 g/L as estimated for TC-HA-1100 and TC-HA-1220, respectively. The inhibition rates reached after 2 h of culture were 45 ± 4 and $30 \pm 6 \%$ for TC-HA-1100 and TC-HA-1220, respectively, in accordance with the inhibition value (about 35 %) expected from similar TC concentrations and M63G culture medium (Supporting Information, Fig. 3). Besides, switching from lag to stationary bacterial growth phase after 8 h of culture may have led to a change in the bacterial susceptibility to the TC antibiotics, as it was already reported in the literature for many antibiotics classes [64–66]. This might have resulted in the loss of bacterial population observed in the presence of TC-HA DDS after 8 h of culture (Fig. 3d) and have thus

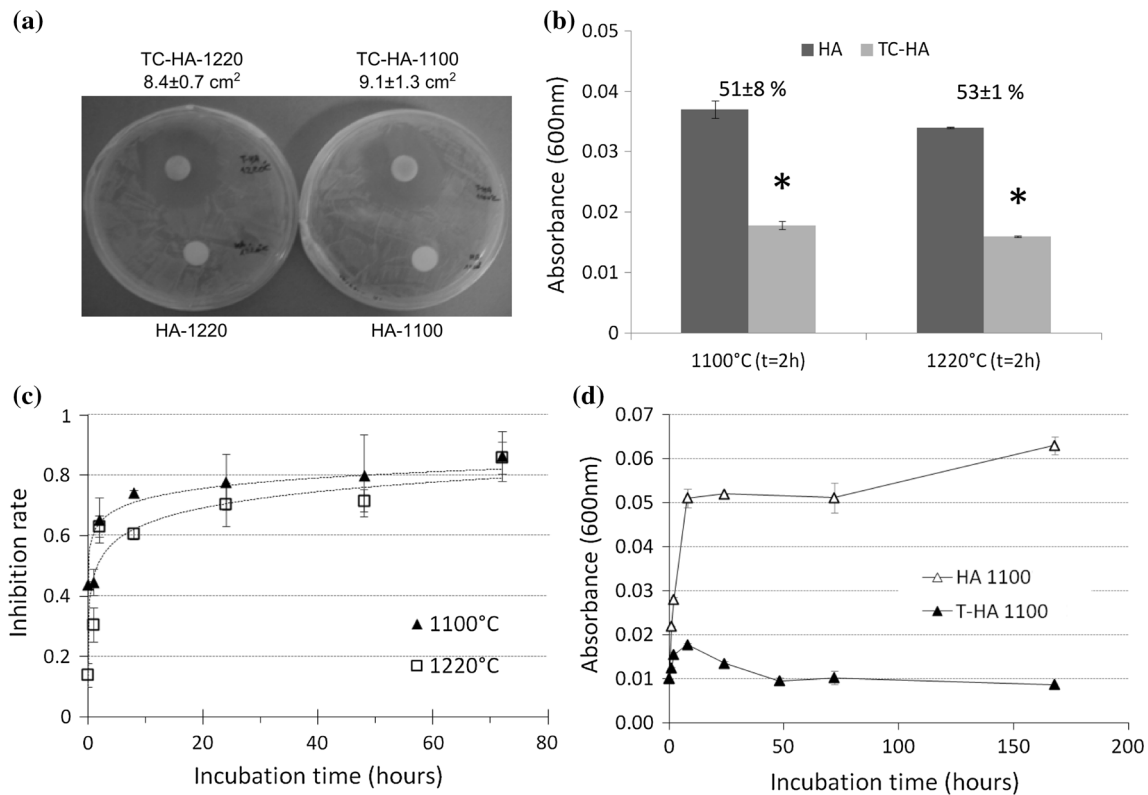


Figure 3 Antibacterial properties of HA and HA-TC DDS on bacteria living in the near material surroundings, on semi-solid nutritive agar plates (a) or in liquid culture medium (i.e. planktonic bacteria) (b, c). a Photographs of TC-HA and HA DDS samples placed onto agar inoculated plates (“diffusion test”). Measurements of inhibition areas around TC-HA-1100 and TC-HA-1220 DDS samples are reported. b Bacteria concentrations measured for HA-1100, HA-1220, TC-HA-1100, and TC-HA-1220 samples

after 2 h of incubation in 4 mL of medium, and corresponding Inh_{plank1} inhibition rates. *significantly different from HA ($\alpha < 0.01$). c Kinetic evolution of bacterial growth inhibition resulting from TC-release calculated from bacteria concentrations measured for HA and TC-HA DDS samples after from 1 h to 1 week incubation time in 22 mL of liquid medium. d Evolution of bacteria population in the presence of HA-1100 and TC-HA-1100 DDS samples during 1 week (168 h).

provided the slight increase of inhibition highlighted in the second period of the experiment.

Inhibition rates measured throughout the kinetics experiments (Inh_{plank2}) reveal higher antimicrobial efficiency of TC-HA-1100 DDS than TC-HA-1220, confirming the results obtained on semi-solid nutritive medium but in contrast to measurements performed in 4 mL of liquid medium (Inh_{plank1}). The difference may result from the methodology used for sampling bacterial suspensions for further measurements of bacteria concentration. Indeed, the supernatants of samples incubated in 4 mL are almost completely removed through several rinsing, leading to harvesting quasi all non-adherent bacteria (Supporting Information, Fig. 2). In contrast, kinetics experiments consist in the removal of small volumes of the bacterial suspension at several specific times,

leading to harvest the fraction of non-adherent bacteria population living in the close surroundings of the material surface e.g. non-adherent bacteria confined in the HA pores. In agreement, bacteria concentrations measured after 2 h of incubation in 4 mL medium are higher than those determined with the kinetics assay for all the HA-1100, HA-1220, TC-HA-1100 and TC-HA-1220 samples (Supporting Information, Table 1). In addition, bacteria concentrations are similar for HA-1100 and HA-1220 when measured in 4 mL assay, while they are in larger number with HA-1100 than with HA-1220 in kinetics experiments for the same incubation time. This difference between both types of HA matrix in the absence of rinsing steps suggests that bacteria free-living in the near material surroundings are in a higher amount with HA-1220 than with HA-1100, which may be

attributed to the difference in pore size of both matrices. Indeed, pore size in HA-1220 matrix (average of $1.4\ \mu\text{m}$) is in better accordance with *E. coli* size ($\sim 1\ \mu\text{m} \times 3\ \mu\text{m}$ as reported in the literature [67] and $2.3 \pm 0.5\ \mu\text{m} \times 1.4 \pm 0.2\ \mu\text{m}$ as measured by our group with fluorescence confocal microscope [68]) than in HA-1100 matrix (average of $1.1\ \mu\text{m}$) (Table 1). Based on the pore size distribution depicted in Fig. 1, it can be estimated that pores with diameter larger than $1.5\ \mu\text{m}$ are rare in HA-1100 material but frequent ($\sim 50\%$ of all pores) in HA-1220 material. This may have enhanced the probability for bacteria to enter pores at the HA-1220 material surface compared to those present at the HA-1100 surface, in agreement with results already reported in the literature regarding impact of surface microtopography on bacterial retention [69–71].

Bacterial attachment

Effect of TC-loaded DDS on short-term material colonization by bacteria was assessed by confocal laser scanning microscopy both in reflection and in fluorescence modes. Bacteria were preferentially observed in pores, with individual bacteria being in topographic features with size similar to bacterial one (Fig. 4a) and bacterial colonies in pore with larger size (Fig. 4b). In accordance with the literature, this was attributed to the retention of individual adhered bacteria in the protected locations formed by pores fitting with the cell size [69, 72–74] and their potential proliferation until colonies were filling the confined space of the pores [74, 75]. This resulted in a heterogeneous distribution of bacteria throughout the samples, as displayed by the high deviation in the bacteria number values extracted from CLSM micrographs. In the antibacterial application point of view, this may compromise the colonization of material surface by the pioneer bacteria retained in pores by preventing the spreading of their clones as suggested by Huysman et al. and Wang et al. [74, 75]. In addition, amounts of adhered bacteria, determined after 2 h of incubation in culture medium on randomly chosen locations of the sample surfaces, show a colonization being significantly reduced on both TC-HA-1100 ($\alpha < 0.01$) and TC-HA-1220 DDS ($\alpha < 0.01$) compared to colonization on HA matrix without TC (Fig. 4c). This result agreed complementary assays consisting in harvesting adhered bacteria by printing the top side of DDS samples onto an agar nutritive plate

(Supporting Information, Fig. 4). On the other hand, variations of up to 41 % of the surface concentration were observed on CLSM micrographs between samples of the same material type, probably resulting from the high heterogeneity of colonization already reported and leading to high deviations in the inhibition rates determined for both HA-1100 and HA-1220 materials (Supporting Information, Table 1). Despite of this reservation, however, the quantity of TC that was loaded in HA-1100 and HA-1220 matrices was shown to be in capacity to inhibit 56 ± 34 and $30 \pm 18\%$ of bacterial adhesion, respectively.

HA-1100 and HA-1220 DDS did not differ in term of bacteria amount adhered on their surface. This suggests that, even if difference in pore size of HA-1100 and HA-1220 may have affected confinement of planktonic bacteria in pores as discussed above, the slightly higher population confined in HA-1220 pores compared to HA-1100 pores was not sufficient to significantly affect the extend of short-term colonization on DDS. In contrast, the reduction in bacterial colonization provided by TC-loaded DDS compared to TC-free DDS differed according to the HA matrix type. Colonization was significantly less on TC-HA-1100 than on TC-HA-1220 DDS, in agreement with the loaded quantity of TC and in accordance to the higher inhibition effect provided by TC-HA-1100 DDS onto planktonic bacteria compared to TC-HA-1220 DDS.

Protein adsorption

Biomolecules of the cell culture medium were expected to adsorb onto DDS and to potentially affect release and antibacterial properties of the DDS. This was tested during perfusion in adequate bioreactors [50]. XPS analysis of the ceramics sample surfaces, performed before and after perfusion in bioreactors, demonstrated the formation of a protein coating, which led to hide the originate composition of the sample surface characterized by $\text{Ca}2p\%$, $\text{P}2p\%$ and $\text{O}1s\%$ components (Supporting Information, Table 2). Weak quantities of $\text{N}1s\%$, $\text{Na}1s\%$ and $\text{Cl}2p\%$ components detected on TC-HA samples before protein adsorption were attributed to TC and its initial buffer, while the slight increases of $\text{Na}1s\%$ and $\text{Cl}2p\%$ components on HA samples after perfusion experiments were attributed to mineral constituents of the culture medium used for adsorption experiments. In the same time, surface content in $\text{C}1s\%$ and $\text{N}1s\%$

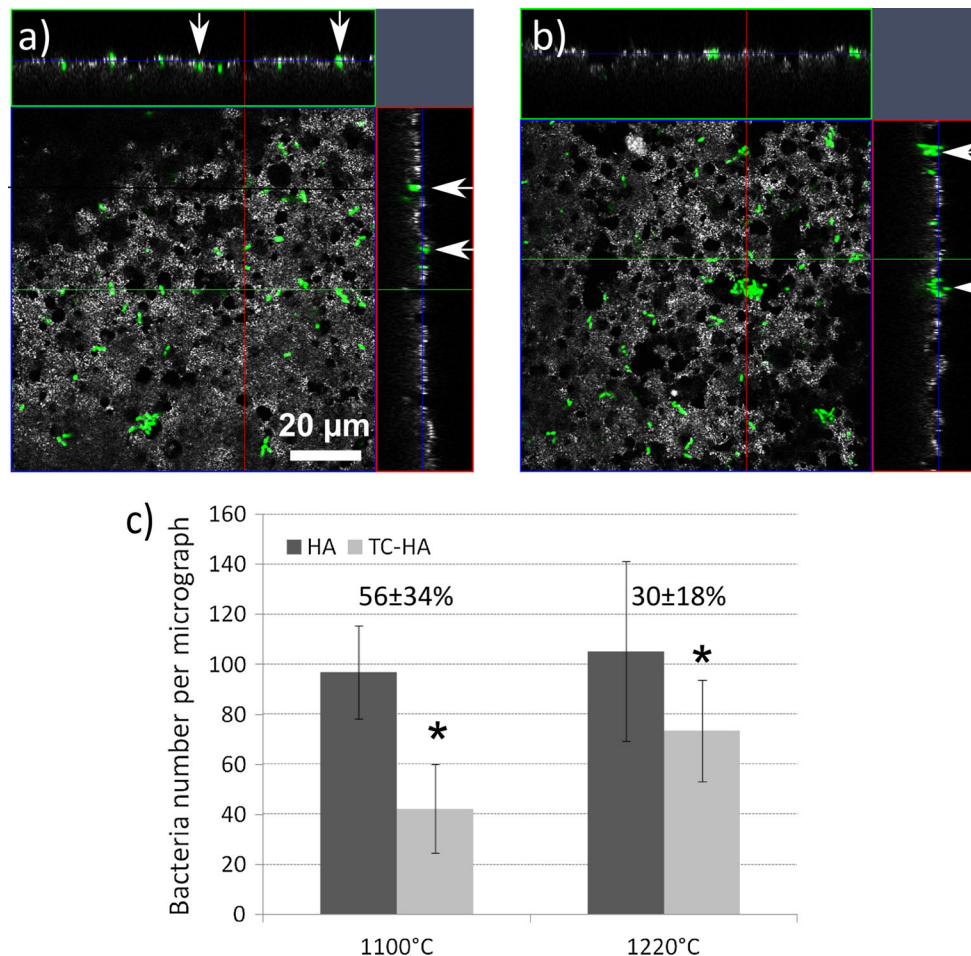


Figure 4 Colonization of HA and TC-HA DDS samples after 2 h of incubation in culture medium. **a** CLSM image showing HA material surface and bacterial colonization observed by reflection and fluorescence modes. Bacteria were labelled using Syto9[®] (Molecular Probes) prior to observation. White arrows show single cells (**a**) and bacterial colonies (**b**) in topographical features of different sizes. **c** Number of adherent bacteria, and corresponding

Inh_{adh} inhibition rate, determined by image analysis on CLSM micrographs. Bacteria were labelled using Syto9[®] (Molecular Probes) prior to observation. Image treatment and image analysis were conducted with ImageJ [47] and CellC software [48], respectively. *significantly different from corresponding HA ($\alpha < 0.01$).

Table 3 Amounts of protein adsorbed on porous HA and TC-HA DDS samples after perfusion with cell culture medium in bioreactors

	HA-1100	HA-1220	TC-HA-1100	TC-HA-1220
Amount of proteins adsorbed per sample (~65 mg of HA)	5 ± 3 mg	3 ± 2 mg	5 ± 3 mg	3 ± 1 mg

components raises in agreement with the presence of biological organic compounds such as proteins.

Quantity of adsorbed proteins was indirectly evaluated by dosing proteins of the culture medium before and after perfusion of HA and TC-HA materials. Similar amounts of adsorbed proteins were measured for HA and the corresponding TC-HA material showing that protein adsorption was not

significantly affected by the presence of TC on the DDS surface (Table 3). Ranging from 45 to 75 mg of proteins per g of HA, they are in agreement with results previously reported for BSA used at similar concentrations as in the present study (i.e. about 2 mg/mL as compared to 10 % FBS in the culture medium) [76–78]. Furthermore, significantly more proteins were adsorbed on HA-1100 and TC-HA-

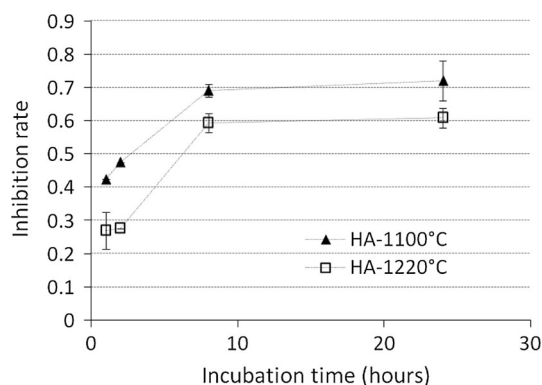


Figure 5 Growth inhibition rates of TC-HA-1100 and TC-HA-1220 materials after previous adsorption of proteins realized in perfusion bioreactors. Concentrations of bacteria were measured in 22 mL medium for 1, 2, 8 and 24 h.

1100 compared to HA-1220 and TC-HA-1220. This is consistent with the higher porosity and porous volume measured in HA-1100 materials compared to HA-1220 ones, which is expected to favour the adsorption of a larger amount of proteins in HA-1100 derived-DDS due to more surface area exposed to proteins [14, 79].

Antibacterial efficiencies onto planktonic bacteria were not modified after adsorption of proteins. They were measured for incubation times from 1 to 24 h for both HA-1100 and HA-1220 matrices, resulting in inhibition rates of planktonic bacteria equivalent to without protein adsorption (Fig. 5) and reaching about 70 and 60 % after 24 h of culture for HA-1100 and HA-1220, respectively. As for materials without protein conditioning, and despite more proteins adsorbed on HA-1100 DDS samples, HA-1100-based DDS offered higher inhibition efficiency on planktonic bacterial population compared to HA-1220 DDS. This may be explained by the presence of proteins adsorbed as aggregates rather than continuous layer as it has been already reported in the literature [78]. Proteins might not completely screen the HA surface and therefore might only slightly or even not prevent the release of TC. Such slight changes in the TC concentration released from DDS may lead to weak, undetectable changes in the bacterial growth.

Finally, TC-HA-1100 matrix was showed to uptake higher doses of TC and to release them in a faster way than TC-HA-1220 matrix, in agreement with the porous structure and especially in relation with the presence of pore interconnections. Accordingly, TC-HA-1100 DDS displayed better antibacterial

properties than TC-HA-1220 DDS in terms of the inhibition of bacteria growth, both in suspension and for bacteria adhered on the material. Nevertheless, and in spite of its satisfying stability observed during the physical–chemical and antibacterial characterization studies, TC-HA-1100 displayed a higher sensitivity to multiple manipulations than TC-HA-1220, due to the weaker resistance to mechanical strains of pore walls, enriched in interconnections and therefore less cohesive compared to HA-1220 bioceramic. Accordingly, TC-HA-1220 DDS were preferred for the experiments with mammalian cells and, further, for applications in *in vivo* conditions.

In vitro biocompatibility for eukaryotic cells

Biocompatibility of HA and TC-HA DDS was tested by culturing MC3T3-E1 cells in media previously immersing DDS samples for 5 days (i.e. allowing total TC release). Proliferation of MC3T3-E1 cells in material-free media was similar to both extraction media of the positive control (i.e. Thermanox[®] coverslip) and media extracted from HA and TC-HA DDS (Fig. 6a), clearly demonstrating the absence of *in vitro* toxicity of the quantity of TC released from TC-HA DDS.

Cell adhesion and proliferation on both HA and TC-HA DDS were also investigated by analysing cell number, viability and morphology (cell area and cell circularity) after 6, 24 and 72 h of culture on the DDS samples (Fig. 6b, c). CLSM micrographs display slightly less adhered cells on HA and TC-HA compared to positive control (Fig. 6d), which is attributed to the surface morphology of the porous HA matrices in agreement to results reported in the literature regarding cell adhesion and proliferation on microstructured surfaces compared to flat surfaces [80]. In contrast, chemical properties of HA are known to be favourable to cell attachment and development [81]. The slightly less quantity of cells was accompanied by the reduction of cell size on HA and TC-HA compared to positive control (Fig. 6e) but similar cell viability and circularity (Fig. 6d, e), suggesting impact of the surface morphology onto cell spreading without any significant cytotoxic effect of the material. Accordingly, number, viability, size and circularity of cells were significantly different on the negative control.

Importantly, any difference was observed between HA and TC-HA DDS in terms of number, size,

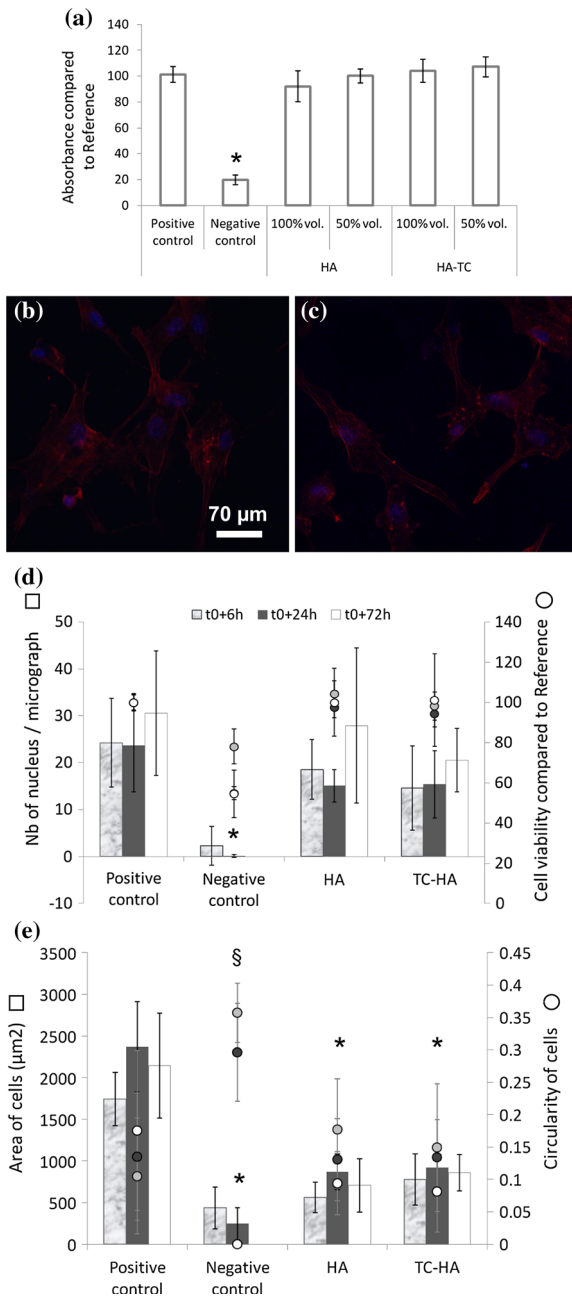


Figure 6 In vitro biocompatibility of HA-1220 and TC-HA-1220 (4.2 ± 0.8 mg TC/g HA) DDS samples. **a** MC3T3-E1 cell proliferation in extraction media of positive control (Thermanox[®]), negative control (Phenol 25 %), HA and TC-HA DDS compared to fresh medium without material (Reference). *significantly different to Reference ($\alpha < 0.01$). **b**, **c** CLSM micrographs of MC3T3-E1 cell after 24 h culture on HA and TC-HA DDS samples, respectively. **d** Number of cell nucleus and cell viability as determined on CLSM micrographs and by PrestoBlue[®] labelling, respectively. **e** Average area and circularity of cells as determined on CLSM micrographs. * and § significantly different to Positive control, for bars and circles, respectively ($\alpha < 0.01$).

circularity and viability of cells, demonstrating the in vitro compatibility of the TC-loaded quantity with the tested eukaryotic cells. This is in agreement with results reported in the literature for similar quantities of TC on mesenchymal cells comparable to MC3T3-E1 cells [82].

Finally, the present results demonstrate the possibility to provide and control antibacterial effects of antibiotics- and HA-bioceramics-based DDS whilst maintaining biocompatible properties for eukaryotic cells. Nevertheless, since anti-infectious efficiency of similar quantities of TC is expected to be modified i.e. reduced in real in vivo conditions of use and even if good accordance of efficient doses in in vivo and in vitro conditions has already been reported in the literature [1, 83], TC quantity in DDS will probably need to be adapted for in vivo use to fit the necessary, favourable antibacterial versus biocompatible properties balance. In vivo studies with this aim are currently under development. Also aiming at the improvement of the DDS performance in in vivo conditions of use, similar DDS with other antibiotics such as vancomycin are under investigation.

Conclusion

New antibiotics DDS based on HA ceramic matrix of controlled porosity were successfully developed by a colloidal heterocoagulation approach using hydroxyapatite nanoparticles and polymethylmethacrylate microspheres. With constant size of microspheres and HA/PMMA ratio, the sintering temperature allowed the modulation of interconnections porosity and volume, then allowing the loading of antibiotics in quantity dependent on matrix porosity. The release and antibacterial properties of DDS of two different porosities were investigated, demonstrating the possibility to control the loaded quantity of antibiotics, and therefore their antibacterial efficiency by varying the matrix porosity. Release kinetics is also slightly affected by changes in ceramic porosity, given the moderate porosity differences induced by varying the sintering temperature. DDS loaded with TC were also shown to provide biocompatible properties for MC3T3 eukaryotic cells in in vitro conditions. Adaptation of TC-loaded quantities for use in in vivo conditions is the subject of further investigations. In addition, similar DDS with alternative antibiotics are currently under evaluation in in vivo conditions.

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