Relevance of the sterilization-induced effects on the properties of different hydroxyapatite nanoparticles and assessment of the osteoblastic cell response

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Hydroxyapatite (Hap) is a calcium phosphate with a chemical formula that closely resembles that of the mineral constituents found in hard tissues, thereby explaining its natural biocompatibility and wide biomedical use. Nanostructured Hap materials appear to present a good performance in bone tissue applications because of their ability to mimic the dimensions of bone components. However, bone cell response to individual nanoparticles and/or nanoparticle aggregates lost from these materials is largely unknown and shows great variability. This work addresses the preparation and characterization of two different Hap nanoparticles and their interaction with osteoblastic cells. Hap particles were produced by a wet chemical synthesis (WCS) at 37°C and by hydrothermal synthesis (HS) at 180°C. As the ultimate in vivo applications require a sterilization step, the synthesized particles were characterized ‘as prepared’ and after sterilization (autoclaving, 120°C, 20 min). WCS and HS particles differ in their morphological (size and shape) and physicochemical properties. The sterilization modified markedly the shape, size and aggregation state of WCS nanoparticles. Both particles were readily internalized by osteoblastic cells by endocytosis, and showed a low intracellular dissolution rate. Concentrations of WCS and HS particles less than 500 μg ml−1 did not affect cell proliferation, F-actin cytoskeleton organization and apoptosis rate and increased the gene expression of alkaline phosphatase and BMP-2. The two particles presented some differences in the elicited cell response. In conclusion, WCS and HS particles might exhibit an interesting profile for bone tissue applications. Results suggest the relevance of a proper particle characterization, and the interest of an individual nanoparticle targeted research.

Keywords: hydroxyapatite nanoparticles; physicochemical properties; autoclave sterilization; osteoblastic response

1. INTRODUCTION

Hydroxyapatite (Hap) is a calcium phosphate (CaP), the chemical formula, Ca_{10}(PO_{4})_{6}(OH)_{2}, of which closely resembles that of the mineral constituents found in hard tissue (i.e. teeth and bones), thereby explaining its natural biocompatibility and wide medical use in implant fabrication, scaffolds for tissue engineering, materials for orthopaedic and dental applications and maxillofacial reconstruction [1,2]. It has been recognized that the genesis of the bone CaP mineral phase implies a deposition process mediated by a living tissue, consisting of CaP synthesis, resorption and replacement under the action of living cells [3]. This dynamic process modulates the composition and structure of the bone CaP mineral phase, which are thus space and time variable, reflecting the variability of body metabolism and the specificity of bone local cellular functions [3]. As a consequence, bone displays a hierarchic structure extending from macroscopic to nanometric scale. The bone mineral phase consists of nanosized crystals of non-stoichiometric Hap having a needle- or plate-like shape, which is typically 20–80 nm long and 2–5 nm thick, densely packed into collagen fibrils [4].
Prompted by the fast nanotechnology developments, several attempts to mimic the biological bone Hap nanoparticle morphology are found in literature. A variety of methods have been proposed to synthesize Hap nanosized particles, including co-precipitation, micro-emulsion, sol gel and hydrothermal synthesis, giving rise to a wide range of nanoparticles [5–9]. Films, granules, composite scaffolds, dense or porous bulk or materials manufactured with Hap nanoparticles exhibit a surface with improved osteoblastic activity and new bone formation, suggesting a clear advantage in bone tissue applications compared with conventional Hap microstructured materials [9–12]. The possibility of such nanostructured materials undergoing some kind of injury or fragmentation during or after the application process with subsequent loss of individual nanoparticles and/or nanoparticle aggregates to surrounding bone tissue is poorly documented, but of utmost relevance [9–12].

Cell response to the nanoparticles differs significantly from that of the bulk materials, because of their specific characteristics, namely the high surface area to volume ratio and the physicochemical surface properties [12,13]. Studies on the interaction of Hap nanoparticles with several cell types [14–22] show a great variability in the elicited response, which is not surprising considering the wide versatility and variability of the physicochemical properties of the tested particles, the cell type and density and the differences in the experimental protocols. Results of these studies evidenced the difficulties in relating the physicochemical properties of Hap nanoparticles and the cell behaviour, and suggested that the cell response is determined by the combined interaction of multi-nanoparticle characteristics. In this context, individual nanoHap particle targeted research is relevant for mapping the specific nanoparticle route and its cellular interaction. Also, this would add information on the cytotoxicity, biostability and functional properties of nanoHap [10,12,23], and other potential applications of nanoHap such as those related to the drug delivery efficiency of Hap-based carriers [2].

The present work is focused on the preparation of two types of Hap nanosized particles displaying distinct physicochemical properties. Inspired by the critical physiological role of citric acid on bone apatite nanocrystal size control and stabilization [24,25], we have envisaged the citrate-assisted growth of Hap nanoparticles as a promising synthesis technique in terms of particle morphology control. Considering the dynamic nature of nanoparticle properties, special attention was given to the particles’ characterization. They were assessed ‘as prepared’ and also after being sterilized by autoclaving. In addition, the sterilized particles, the true targets for biological studies and applications, were evaluated for their effects on human osteoblastic cells.

2. MATERIAL AND METHODS

2.1. Hydrothermal and wet chemical synthesis of Hap nanoparticles

Two methods were used to synthesize Hap nanoparticles: hydrothermal synthesis (HS), carried out at 180°C, and wet chemical synthesis (WCS), performed at 37°C. The necessary supersaturated solutions for both methods were prepared by mixing citric acid (Riedel-de-Haën, 99.5% purity), calcium nitrate (Riedel-de-Haën, 99% purity) and ammonium hydrogen phosphate (Merck, 99% purity) as follows: an aqueous citric acid solution (0.6 M) was added with small amounts of ammonia (Riedel-de-Haën) (25 vol.%) for the pH adjustment up to 8.1, and then mixed with appropriate amounts of calcium nitrate solution (0.2 M), prior to the addition of ammonium hydrogen phosphate solution (0.2 M). The mixture of Ca and citric acid solution provides the conditions for citrate complexation to take place before the subsequent phosphate addition, thus inhibiting immediate precipitation [7]. The mixture was prepared under continuous stirring, at room temperature. The resulting homogeneous (calcium/citrate/phosphate) solution was split in two parts: one part was put in a sealed glass vessel and maintained at 37°C in a thermostatic water bath for 24 h (wet chemical synthesis, WCS); the other part was transferred to a tightly sealed Teflon vessel in a stainless steel autoclave and kept at 180°C in an oven during 24 h (hydrothermal synthesis, HS).

The pressure developed inside the autoclave during hydrothermal processing was estimated as roughly corresponding to the pressure of saturated water vapour at 180°C, i.e. approximately 10 bars. After 24 h, the two vessels (WCS, HS) were cooled and the precipitated particles were collected by filtering the suspensions through a 0.22 μm millipore filter and washed repeatedly with deionized water. The obtained powders were dried in a desiccator. Finally, the so prepared particles were sterilized in a steel autoclave at 120°C during 20 min, according to a standard procedure in biomedical applications. After being submitted to sterilization, the particles obtained by HS and WCS methods were here labelled as HSpst and WCPs, respectively.

2.2. Characterization of Hap nanoparticles

The microstructure and morphological features of Hap particles, before and after sterilization, were evaluated with a Hitachi H-9000-NA transmission electron microscopy (TEM) operating at 200 kV. The particle samples for TEM observation were prepared by dropping an ethanolic suspension of the particles kept under ultrasonic stirring onto a formvar-coated copper grid. The grid was subsequently air-dried for TEM observation.

The specific surface area of the precipitated particles was determined by N2 adsorption using a Micromeritics—Gemini 2370 v. 5 equipment. The samples were thoroughly degassed for 24 h at 120°C before nitrogen adsorption, except WCS particles, which were degassed at 60°C. The particle-specific surface area was determined based on the multipoint Brunauer—Emmett—Teller isotherm (BET).

The crystalline phases of the precipitated nanoHap particles were identified by powder X-ray diffraction (XRD) analysis using an X-ray diffractometer, model Rigaku PMG-VA with a Cu-Kα incident radiation (1.5405 Å). The diffraction patterns were recorded at room temperature over the 2θ range of 10°–70° at 3° per min in a continuous mode. Crystallite sizes were
estimated from the X-ray diffractograms using the Scherrer formula \[ D = \frac{k \Lambda}{\beta \cos \theta} \text{, where } D \text{ is the crystallite size (Å) estimated using the reflections (002) or (004), } k \text{ is a shape factor equal to 0.9, } \lambda \text{ is the X-ray wavelength (1.5405 Å), } \theta \text{ is the diffraction angle related to the reflections 002 (} \theta = 12.92 \text{) or 004 (} \theta = 26.59 \text{), respectively, and } \beta_1/2, \text{ expressed in radians, is defined as } \beta_1/2 = (B_2 - B_1)/2, B \text{ being the diffraction peak width at half height and } b \text{ the natural width of the instrument. The calcium phosphate ratio (Ca/P) of the synthesized particles was also evaluated by energy dispersive X-ray analysis (EDX) spectroscopy using an Oxford INCA instruments EDX spectrometer coupled to a scanning electron microscope at 15 kV accelerating voltage.}

Fourier transform infrared spectroscopy (FTIR) was performed for identifying the functional groups, using a Mattson galaxy 3020 spectrophotometer. The test samples were prepared by mixing approximately 2 mg of Hap powder with approximately 300 mg of spectroscopic-grade KBr (Merck) and forming the mixture into a disc shape. The infrared spectra were recorded in a transmittance mode in the region of 4000–400 cm⁻¹ with a resolution of 4 cm⁻¹.

Thermo-gravimetric analyses were conducted on WCS and HS particles, prior and post sterilization, to follow their thermal behaviour. The analyses were carried from room temperature up to 1000°C in a SetaramLabeyes 1600 equipment using a heating rate of 10°C min⁻¹ under N₂ flow.

The measurement of the electrophoretic mobility of the particles in aqueous suspensions was performed at 25°C, using a Malvern equipment with zetasizer nano series 6.00 software. A small amount of the particles was suspended in a 2 × 10⁻⁷ M KCl solution in order to ensure a constant electrical double-layer thickness and then allowed to equilibrate for 2 h before measurement.

2.3. Interaction of Hap nanoparticles with MG63 osteoblast-like cells

MG63 osteoblast-like cells (ATCC number CRL-1427) were cultured in α-minimal essential medium containing 10 per cent foetal bovine serum, 50 µg ml⁻¹ ascorbic acid, 100 IU ml⁻¹ penicillin, 2.5 µg ml⁻¹ streptomycin and 2.5 µg ml⁻¹ fungizone, at 37°C in a humidified atmosphere of 5 per cent CO₂ in air. For subculture, adherent cells were enzymatically released (0.05% trypsin, 0.25% EDTA) and the cell suspension was cultured (10³ cell cm⁻²) in standard polystyrene tissue culture plates. After 24 h, the medium was removed and a fresh medium containing the sterilized WCS or HS Hap nanoparticles was added to the adherent cells. Cultures were maintained for 3 and 6 days, without any change of medium. Cultures performed in the absence of nanoHap were used as control.

Cell response was characterized for cell viability/proliferation in the concentration range 50–5000 µg ml⁻¹. Toxic effects were observed for concentrations more than 500 µg ml⁻¹. Thus, a more detailed cell characterization was performed in the presence of lower levels, 50 and 500 µg ml⁻¹.

2.3.1. Cell viability/proliferation

Cell viability/proliferation was estimated by the MTS assay (CellTitert 96 AQueous Cell Proliferation Assay, 1.9 mg ml⁻¹, Promega Corporation). This method is based in the reduction of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] by the mitochondrial succinic dehydrogenase of proliferating cells, to a yellow-coloured soluble formazan compound. Control cultures and those exposed to nanoHap (50–5000 µg ml⁻¹) were incubated with MTS (20 µl/well, in 96-well plates), during the last 4 h of the culture periods tested (3 and 6 days). The absorbance of the formazan compound was measured at 490 nm in an ELISA reader (Synergy HT, Biotek). Six replicates were set up at each condition.

2.3.2. Immunofluorescent staining of F-actin cytoskeleton filaments and nuclei

Cultures exposed to WCS and HS nanoparticles, 50 and 500 µg ml⁻¹, were fixed (4% formaldehyde methanol, 15 min), permeabilized with 0.1 per cent Triton (5 min, RT) and incubated in 10 mg ml⁻¹ bovine serum albumin (BSA, 1 h, RT) with 100 µg ml⁻¹ RNase. Following, F-actin filaments were stained with 480 Alexa-Fluor-conjugated phalloidin (1 : 100, 1 h, RT) and nuclei were counterstained with 10 µg ml⁻¹ propidium iodide (10 min, RT). Labelled cultures were mounted in Vectashield and examined with a Leica SP2 A0BS (Leica Microsystems) microscope.

2.3.3. Transmission electron microscopy

Cultures exposed to WCS and HS nanoparticles, 50 and 500 µg ml⁻¹, for 3 h, 24 h and 3 days, were processed for TEM. Briefly, adherent cells were enzymatically released (0.05% trypsin, 0.25% EDTA) and the cell suspensions were centrifuged at 2000 g for 10 min. The resulting pellet was fixed with 2.5 per cent glutaraldehyde, postfixed with 1 per cent osmium tetroxide, dehydrated in graded ethanol and later embedded in Epon, using routine methods. Ultra-thin (100 nm) sections mounted in copper grids (300 Mesh) were contrasted with uranyl acetate and lead citrate for TEM analysis (Zeiss EM 10A) at an accelerating voltage 60 kV.

2.3.4. Apoptosis

Adherent 24-h MG63 cells were exposed to WCS or HS Hap nanoparticles, 50 and 500 µg ml⁻¹, for 3 days, and evaluated by flow cytometry for the presence of apoptosis. Cells were enzymatically released (0.05% trypsin, 0.25% EDTA) and approximately 10⁶ cells were processed with the TACS Annexin V-FITC Apoptosis Detection kit following the manufacturer’s instructions. Annexin V, an anticoagulant protein, binds negatively charged phospholipids (phosphatidylserine) on the outer leaflet of the cytoplasmic membrane following phospholipid asymmetry disruption occurring in the early apoptotic process. The combination of annexin V-FITC and propidium iodide allows for the differentiation of early apoptotic cells (annexin V-FITC positive), late apoptotic and/or necrotic cells (annexin V-FITC and propidium iodide positive) and viable cells (unstained).
2.3.5. Gene expression by reverse-transcription polymerase chain reaction

Adherent 24-h MG63 cells were exposed to WCS or HS Hap nanoparticles, 50 and 500 μg ml⁻¹, for 3 days, and evaluated by reverse-transcription polymerase chain reaction (RT-PCR) for the expression of the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and the osteoblastic genes collagen 1 (Col1), alkaline phosphatase (ALP) and bone morphogenetic protein-2 (BMP-2). Total RNA was extracted using RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. The concentration and purity of total RNA in each sample were determined by UV spectrophotometry at 260 nm and by calculating the $A_{260\text{ nm}}/A_{230\text{ nm}}$ ratio. Half a microgram of total cellular RNA from each sample was reverse transcribed and amplified (25 cycles) with the Titan One Tube RT-PCR System (Roche), with an annealing temperature of 55°C. The primers used are listed on table 1. RT-PCR products were electrophoretically separated on a 1% (w/v) agarose gel and subjected to densitometric analysis with ImageJ 1.41 software. Values were normalized to the corresponding GAPDH value.

2.4. Statistical analysis

Triplicate experiments were performed. For each experiment, six replicates were set up. The results are shown as the arithmetic mean ± the standard deviation (± s.d.). Statistical analysis was done by one-way analysis of variance (ANOVA). Statistical differences between groups were determined by the Bonferroni method, with a significance level of $p < 0.05$.

3. RESULTS

3.1. Physicochemical properties of Hap nanoparticles

3.1.1. Transmission electron microscopy: specific surface area

Figure 1a shows TEM images of the nanoHap particles. WCS presented a needle-like shape with a width of a few nanometres and a length of approximately 100 nm. In the cell surroundings, nanoparticles were seen either in the isolated form or as varying size aggregates interacting with the cell membrane. HS and WCS nanoparticles showed to be relatively well dispersed over the grids, though some tendency towards aggregation could be noticed in WCS. Sterilization induced a significant modification of WCS features: besides looking significantly agglomerated, the particles suffered a morphology change as they become thicker after sterilization. HS nanoparticles did not undergo any significant morphology change.

The evolution of the specific surface area of both types of particles is in line with the morphology changes previously referred (table 2). Sterilization did not appreciably affect the specific surface area of HS particles, while a large decrease occurred in WCS, from 171.0 to 22.3 m² g⁻¹. The Ca/P ratio of the prepared particles (table 2) showed negligible deviations from the stoichiometric value (1.68) which did not differentiate significantly the two types of precipitated particles.

3.1.2. X-ray diffraction analysis

Figure 1 shows the XRD patterns of WCS and HS particles, as prepared and after being sterilized. The peaks of the XRD patterns of all the examined specimens can be attributed to hexagonal Hap (space group P6₃/m, $a = b = 9.4320$ Å, $c = 6.8810$ Å) according to the data referenced by JCPDS N° 09-0432. It is worth mentioning that the better defined XRD patterns of HS nanoparticles with sharper peaks indicate HS particles to have higher crystallinity than WCS, which contain some amorphous calcium phosphate phase. Crystallite sizes were estimated for WCS and HS and confirmed larger crystalline dimensions in HS, as shown in table 2. Moreover, it was found that sterilization enhanced WCS crystallinity since WCSSter crystallite sizes were slightly larger when compared with WCS. In spite of this, WCSSter nanoparticles crystallinity still stayed behind that of HSsterol nanoparticles.

3.1.3. Fourier transform infrared spectroscopy

The FTIR spectra of WCS and HS nanoparticles are shown in figure 2. WCS or WCSSter spectra were distinct from those of HS or HSSter particles but no remarkable changes were detected between non-sterilized nanoparticles and their sterilized counterpart. All the examined samples exhibited the main vibration bands normally attributed to Hap. Briefly, the bands detected at 472, 563, 601, 962 cm⁻¹ and in the 1030–1092 cm⁻¹ range are attributed to vibrations of P–O bonds [27]. The peaks at 3570 and 632 cm⁻¹ are assigned to the structural hydroxyl anions [28]. Although common features were also identified in the 1250–1700 cm⁻¹ spectral region where citrate-related bands are normally found, some dissimilar bands were observed which may be attributed to carboxylate groups coordinated to the particle surface in a different manner [6–8,29]. When comparing sterilized and unsterilized particle spectra, no visible differences were detected between WCSSter and WCSSter, but only minor ones between HS and HSSter. These include the shoulder at 1402 cm⁻¹ and the band at 1567 cm⁻¹ related to stretching modes of carboxylate groups coordinated in a monodentate or bidentate manner to Hap surface Ca²⁺ cations [30–36] which

<table>
<thead>
<tr>
<th>Gene</th>
<th>5' Primer</th>
<th>3' Primer</th>
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<tbody>
<tr>
<td>GADPH</td>
<td>5'-CAGGACCAGGTTCACCAACAAAGT-3'</td>
<td>5'-GTGGCAGTGATGGCATGACTGT-3'</td>
</tr>
<tr>
<td>COL1</td>
<td>5'-TCCGGCTCTGCTCCCTCTA-3'</td>
<td>5'-ACCAGCGGACCAGCATCTC-3'</td>
</tr>
<tr>
<td>ALP</td>
<td>5'-ACGTGGCTAAGAATGCTATC-3'</td>
<td>5'-CTGGTAGGCGATGCTCTTA-3'</td>
</tr>
<tr>
<td>BMP-2</td>
<td>5'-GCAATGGCCCTTATCTGTCGAC-3'</td>
<td>5'-GCAATGGCCCTTTATCTGTCGAC-3'</td>
</tr>
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Table 1. Primers used on RT-PCR analysis of MG63 cell cultures.
appeared less intense in HS$_{ster}$. This may indicate a partial loss of adsorbed carboxylate groups by HS particles.

3.1.4. Zeta potential

Figure 3 shows the pH dependence of the zeta potential ($\xi$) for the nanoparticles under analysis. The $\xi$ values were negative in the examined pH range except for HS$_{ster}$, which display positive $\xi$ values for the lowest pH values (pH < 5.4). In addition, it is shown that WCS and WCS$_{ster}$ nanoparticles presented more negative $\xi$ values than HS and HS$_{ster}$ nanoparticles. WCS and WCS$_{ster}$ particles were seen to display close $\xi$ curves in the range 5 < pH < 7 whereas the $\xi$ curve of HS nanoparticles is clearly shifted towards more negative values than HS$_{ster}$.
thereby indicating HS nanoparticle surface charge to become less negative upon sterilization.

### 3.1.5. Thermo-gravimetric analysis

The thermo-gravimetric behaviour (curves not shown) of the synthesized particles revealed weight losses occurring approximately between 25°C and 180°C attributed to adsorbed water and organic losses above 180°C assigned to citrate or citrate-related species. WCS adsorbed water and organic contents were found to be approximately 9 wt% and 21 wt%, respectively, whereas smaller values were detected for HS, i.e. approximately 4 wt% and 7 wt%, respectively. It is worth emphasizing that normalizing organic contents relatively to particle surface area, similar values of 0.123 and 0.127 wt% m⁻² are obtained for WCS and HS, respectively. It was further observed that the normalized organic content variation during sterilization was negligible.

### 3.2. Interaction of Hap nanoparticles with osteoblastic cells

#### 3.2.1. Cell viability/proliferation

NanoHap induced concentration-dependent deleterious effects in the cell viability/proliferation of MG63...
In the cell surroundings, nanoparticles were seen isolated and as varying size aggregates interacting with the cell membrane. Within the aggregates, HS particles maintained their shape, but WCS particles appeared more loose than in the compact aggregates, before contact with the cell culture medium (figures 6 and 7).

Control cells showed a fusiform shape with intact cytoplasmic and nuclear membranes. These cells presented a normal distribution of the intracellular organelles, i.e., nucleus with uniformly dispersed chromatin and clear nucleolus, and the cytoplasm containing randomly distributed organelles and electron-dense granules. Following nanoHap interaction, cells readily internalized the nanoparticles (figures 6 and 7). Uptake was apparently initiated by the emission of pseudopod-like projections towards nanoparticles with further fusion of their membranes, which entraps particles inside endosomes (figures 6 and 7). Several varying size vesicles loaded with particles were observed scattered within the cytoplasm (figures 6 and 7); isolated particles were not detected in the cytosol, cell nucleus or in other organelles. The uptake of the nanoparticles seemed to be time- and concentration-dependent, as cells exposed during 3 days to 500 μg ml⁻¹ WCS or HS nanoHap presented huge loaded vesicles. Also, at these nanoHap levels, several cells were noticed exhibiting characteristic signs of apoptosis, i.e. cell membrane shrinkage with bleb formation, cytoplasm budding with vacuolization and chromatin condensation at the nuclear periphery (figures 6 and 7). The presence of cellular debris was also noticed in WCS and HS cultures, particularly after 3 days of exposure, which probably results from the progressive degeneration of apoptotic bodies. At high magnification, figure 8, images showed the close interaction between the nanoparticles and the cell membrane and the vesicles loaded with clustered particles; after 3 days, a high percentage of particles maintained their rod-like morphology, especially HS nanoHap. However, signs of particle dissolution were noticed, particularly in WCS particles.

3.2.4. Apoptosis. Gene expression of osteoblastic proteins
Cultures exposed to 50 μg ml⁻¹ nanoHap for 3 days presented a low percentage of apoptotic cells, similar to control, as assessed by flow cytometry. However, interaction with 500 μg ml⁻¹ particles increased the number of cells involved in early (around 20%) and late (around 7%) apoptosis. Representative histograms are shown in figure 9a.

RT-PCR analysis showed that WCS and HS particles, 50 and 500 μg ml⁻¹, did not affect the expression of collagen type I but caused significant effects in the expression of ALP and BMP-2, figure 9b. HS particles caused a dosedependent increase in the expression of ALP and BMP-2. WCS particles induced significantly the expression of ALP and BMP-2 at 50 μg ml⁻¹, but decreased values were observed in the presence of 500 μg ml⁻¹.

4. DISCUSSION
In the present study, two types of Hap nanoparticles, WCS and HS, were produced and characterized as...
Figure 5. CLSM images of MG63 cell cultures stained for F-actin cytoskeleton (green) and nucleus (red) and exposed to sterilized WCS and HS Hap nanoparticles, 500 μg ml\(^{-1}\), for 3 and 6 days. Control: absence of nanoparticles. Scale bars, 100 μm.

Figure 6. Representative TEM micrographs of MG63 cell cultures exposed to sterilized WCS Hap nanoparticles, 50 and 500 μg ml\(^{-1}\), during different periods (3 h, 24 h and 3 days). The emission of pseudopods surrounding adjacent nanoparticles is observed in (a) (3 h, 50 μg ml\(^{-1}\)) and (b) (3 h, 500 μg ml\(^{-1}\)); (d) (24 h, 500 μg ml\(^{-1}\)) and (e) (3 days, 500 μg ml\(^{-1}\)), depict clusters of nanoparticles entrapped in endosomes. Apart the entrapped nanoparticles within endosomes, it can be also observed in (c) (24 h, 50 μg ml\(^{-1}\)) cellular debris in intercellular space. A cell with early signals of apoptosis, such as the rounded shape, the cytoplasmic vacuolization and signs of blebs formation is shown in (f) (3 h, 500 μg ml\(^{-1}\)).
NanoHap particles were prepared by a citrate-mediated synthesis [7], taking into account the physiological role of this ion in the bone tissue. Citrate molecules are strongly bound to apatite surfaces, and the bound citrate accounts for 5.5 per cent of the organic matrix.

Figure 7. Representative TEM micrographs of MG63 cell cultures exposed to sterilized HS Hap nanoparticles, 50 and 500 µg ml⁻¹, during different periods (3 h, 24 h and 3 days). (a) (3 h, 50 µg ml⁻¹) and (b) (3 days, 50 µg ml⁻¹) depict the emission of pseudopods surrounding nanoparticles and in (d) (24 h, 500 µg ml⁻¹), (e) (3 days, 50 µg ml⁻¹) and (f) (3 days, 500 µg ml⁻¹) it can be observed clusters of nanoparticles entrapped in endosomes. An apoptotic cell with a rounded shape, condensed chromatin at nuclear periphery, cytoplasmic vacuolization and signs of blebs formation is shown in (c) (24 h, 500 µg ml⁻¹).

Figure 8. High magnification TEM images of MG63 cell cultures exposed to sterilized WCS and HS Hap nanoparticles, 500 µg ml⁻¹, for 24 h. (a,e) Show the close interaction of the nanoparticles with the cell membrane, and (b,f) the intracellular vesicles loaded with the particles. (c,g) A high magnification of (b) and (f), respectively, display the nanoparticles within the intracellular vesicles, showing signs of particle dissolution. (d,h) Display the electron diffraction patterns of the internalized nanoparticles.
matter in bone [24], with approximately one-sixth of the available apatite surface being covered by citrate molecules [25]. This chemical entity appears to have a critical role in crystal thickening and stabilization of the apatite nanocrystals through its carboxylic groups [7,24,25].

WCS and HS nanoparticles presented different morphology and crystallinity features which reflect the temperature and pressure conditions that distinguish the two synthesis methods. Such differences are in line with frequent reports showing that Hap synthesis at high temperature \( (T > 100^\circ C) \) leads to crystalline and well-defined prismatic particles, while lower temperature synthesis generally favours thinner particles with less perceptible contours and lower crystallinity [6,30–32]. Significant changes in the morphology were detected in WCS nanoparticles after sterilization. Exposure of WCS particles to a temperature higher than that of the synthesis, furthermore assisted by a saturated steam atmosphere, allowed the smaller-sized WCS particles to undergo a partial dissolution subsequently followed by a re-precipitation process which contributed to the growth of the existing HAP coherent crystalline regions. The large aggregates observed after sterilization are thus envisaged as resulting from re-precipitation processes that allowed adjacent particles to become chemically bonded. These events would explain the large decrease in WCS-specific surface area and the observed crystallite growth during sterilization. This behaviour is consistent with literature reports showing that a combined high temperature and saturated steam regime promotes the local dissolution of the extremely unstable and

Figure 9. MG63 cell cultures exposed to sterilized WCS and HS Hap nanoparticles, 50 and 500 \( \mu g \text{ ml}^{-1} \), for 3 days. (a) Apoptosis, representative histograms. (b) RT-PCR gene expression of collagen I, ALP and BMP-2; the PCR products were subjected to a densitometric analysis and normalization to the corresponding GAPDH value; asterisks (*) significantly different from control (absence of nanoparticles).
hydrolysable amorphous calcium phosphate phase and its recrystallization as the HAP phase [33,34]. Regarding the steam sterilization behaviour of HS particles, some crystallite size variations are also noticed but with a smaller extent than those of WCS. Since particle size, shape and surface area are maintained practically unchanged during autoclaving it is concluded that dissolution and re-precipitation processes, if any, were negligible.

The different morphology variations suffered by HS and WCS particles during sterilization were accompanied by different trends in surface charge variation: whereas WCS surface charge remained practically unchanged with sterilization in the lower pH range (5 \( \leq \text{pH} \leq 7 \)), HS particles tended to a less negative surface charge. Thermo-gravimetric data revealed equivalent organic amounts per unit of surface area in both WCS and HS particles, either sterilized or unsterilized. However, as evidenced by FTIR results, the configurations of the carboxylate groups adsorbed on the surfaces of WCS (or WCS_{surf}) and HS (or HS_{surf}) particles are different. The citrate contribution to the particle surface charge will be ruled by the amount of free carboxylate groups negatively charged, which in turn is conditioned by the citrate coordination mode to Hap surface. Moreover, it was already reported that citric acid may degrade at \( T > 145 \text{C} \) [35,36] leading to reaction products that include different dehydroxylated or decarboxylated acids and anhydrides depending on the used heating rate and temperature. It is thus hypothesized that the original citrate ions do not exist as adsorbed species on the HS particle surface, but instead as smaller carboxylated ions resulting from citrate ion decomposition under the high temperature and pressure used for Hap hydrothermal synthesis. The above reasoning supports the existence of different citrate-related species on the surface of the two types of particles which contribute differently to their surface charge and zeta potential curves. As WCS particles were less prone to surface charge modifications upon sterilization than HS, and no visible modifications of WCS FTIR spectra occurred with sterilization, the adsorbed citrate ions are thought to behave as a stable buffer layer playing a key role on WCS surface charge maintenance. Regarding HS, as previously referred, the intensity of some carboxylate related bands in FTIR spectrum tended to decrease upon sterilization thus being concluded that a partial desorption of adsorbed carboxylated species might have occurred thereby explaining the small increase of HS_{surf} zeta potential.

Osteoblastic cell response to sterilized WCS and HS particles showed that cell viability/proliferation was affected only at high levels, more than 500 \( \mu \text{g ml}^{-1} \). Levels of 1000 and 5000 \( \mu \text{g ml}^{-1} \) caused deleterious effects that appeared to have resulted from a direct contact toxic effect due to the large amount of particles in the cell environment, a situation that is not expected to reflect the clinical settings. Thus, detailed cell response to the nanoHap particles was assessed for lower levels, 50 and 500 \( \mu \text{g ml}^{-1} \).

Sterilized WCS and HS nanoparticles were seen in the cell culture environment isolated and as big aggregates. Within HS aggregates, individual particles retain the original size and shape, but in WCS aggregates the particles appeared relatively loose compared with that seen before contact with the culture medium, suggesting a partial dissolution which might be related to their lower crystallinity. Culture medium was supplemented with foetal calf serum which contains a variety of proteins and other bioactive molecules that quickly form an adhesion layer on the reactive surface of the nanoparticles, favouring the formation of agglomerates of attached particles, as also seen in previous studies involving the interaction of nanoHap with several cell types [16,18,37]. The biological relevance of surface adsorbed proteins has been highlighted and the ‘protein-corona’, a durable and stabilizing coating of nanoparticles monomers and/or particles assemblies, is considered the effective unit of interest in the cell nanomaterial interaction [38–41]. Regarding nanoHap particles, surface adsorbed serum proteins was reported to alter the surface biochemistry and also properties such as size, surface charge, aggregation behaviour and solubility [22,37]. WCS and HS particles were both negatively charged at physiological pH, but WCS particles exhibited much lower zeta potential values, which are expected to influence the composition, exchange events and stability of this protein corona [13,38–41]. However, as the zeta potential of the resulting particle–protein complex is not known, the relevance of the different surface charge of WCS and HS particles on cell uptake and response cannot be inferred.

TEM images showed a close interaction of WCS and HS nanoHap with the cell membrane, followed by an internalization process by endocytosis pathways, as expected for the uptake of nanoparticles [13,42]. Images suggested a time- and dose-dependent uptake, but a great variability was observed on the percentage of cells showing internalized particles and in the number and size of the loaded vesicles. This might be related to limitations of the experimental protocol, namely the heterogeneity of the cell–particle interactions in the cell culture environment. Previously, uptake of nanoHap was reported in periodontal ligament cells [16], liver cancer cells [18], macrophages [20] and mesenchymal stem cells [43] interacting with isolated particles, or in rabbit osteoblastic cells and U2OS cells [17] and MG63 cells [19] growing over nanoparticles films. These studies suggested that the particle uptake depends on the cell type [17], the cell density [42] and, in the same cell type, the particle properties [20].

Regarding the fate of internalized lysosomic Hap nanoparticles, high magnification TEM images suggested some particle dissolution, which is expected due to the acidic environment of the lysosome, and as observed previously in macrophages [20] and osteoblasts [21]. The dissolution process may induce the saturation of the lysosome environment regarding calcium, phosphate and hydroxide ions and the elevation of the local pH [44], and both events may contribute to inhibit further Hap dissolution [20]. This might explain the relatively low degradability of WCS and HS nanoHap particles after 3 days. Nevertheless, particle dissolution was apparent, more pronounced in WCS nanoHap, which might result from its lower crystallinity.
The interaction of MG63 cells with low levels of WCS and HS nanoHap, 50 μg ml⁻¹, results in a rapid internalization without affecting cell viability/proliferation, the overall cell morphology and the percentage of apoptosis. In addition, both WCS and HS particles caused a significant increase in the gene expression of the osteoblastic markers ALP and BMP-2. This is in line with previous studies showing that particulate nanoHap exhibits functional properties, namely by modulating proliferation [22] and the expression of several genes in osteoblastic cells, including collagen type I [22], ALP [21] and increasing ALP activity in periodontal ligament cells [16]. However, at high levels (500 μg ml⁻¹), the presence of intracellular WCS- or HS-loaded vesicles induced alterations on the overall cell morphology, changes on the F-actin cytoskeleton and apoptotic events. These deleterious effects were probably due to the big filled vesicles and associated high mechanical strains, which damaged the cell structure, causing cell degeneration. Similar toxic effects were reported before, namely apoptosis in MG63 cells growing over nanoHap films [19] and cytoskeleton alterations in endothelial cells [14,15]. In addition to the mechanical mediated effects, the partial degradation/dissolution of the particles within the lysosome and the subsequent diffusion of calcium and phosphate ions to the cytoplasm have been suggested as playing a relevant role in the modulation of cell behaviour by nanoHap particles. In the cytoplasm, calcium concentration is lower than 200 nM but it can attain very high levels, 400–600 μM, within the lysosome [44], and even minor changes in cytoplasmic calcium have significant effects in cell behaviour, as this ion has a major role as a second messenger by modulating a variety of cellular functions [45]. However, information on the intracellular trafficking and signalling pathways is lacking.

WCS and HS particles displayed some differences on the induced cell response, namely in the cytotoxic potential and in the gene expression of osteoblastic markers. Main differences in the physicochemical properties of the sterilized particles were the more negative zeta potential and the lower crystallinity of WCS nanoHap, conditioning their interaction with proteins, particle aggregation, dissolution rate, uptake, all affecting cell response [38–42]. Particles also differed on the amount of adsorbed citrate ions, which may be of biological significance, considering that citrate is an important metal ion binder, with a high affinity for calcium, being involved in key physiological functions and intracellular metabolic events, including in the bone tissue [24,25,46–49]. However, no single properties were identified to explain the differences seen in the behaviour of MG63 cells. This observation is in line with previous studies that also showed a great variability in the cell response to nanoHap [13–20]. In spite of the variability in the experimental conditions used, the authors suggested that the elicited response depends on the combined interaction of multiple nanoparticles characteristics, leading to a different profile of protein adsorption, particle aggregation, uptake, degradation/ dissolution and intracellular trafficking events, suggesting the interest of individual nanoparticle targeted research.

5. CONCLUSION

WCS and HS nanoHap particles were distinguished by their morphological attributes, i.e. size and shape, and also by their different surface chemical condition reflected in both zeta potential curves and FTIR spectra. Additionally, sterilization is seen to modify markedly the shape, size and aggregation state of WCS nanoparticles, the high surface area of which suffered a severe reduction during autoclaving.

Sterilized WCS and HS nanoHap particles were readily internalized by MG63 osteoblastic cells, by an endocytic pathway, and exhibited some differences in the elicited cell response. Low levels, not associated with deleterious effects in the cell viability/proliferation, F-actin cytoskeleton organization and apoptosis rate, were able to increase the expression of ALP and BMP-2. Deleterious effects on cell behaviour were observed only at high concentrations, probably because of the vesicular entrapment of the particles and their low degradability. Due to the low toxicity and the potential for the modulation of the osteoblastic cell behavior, WCS and HS nanoHap particles are likely to exhibit an interesting profile for bone tissue applications.

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