Electronic Supplementary Material (ESI) for CrystEngComm

Electronic Supporting Information

Producing Nanotubes of Biocompatible Nano-Hydroxyapatite by Continuous Hydrothermal Synthesis

Edward Lester,[a,b] Selina V. Y. Tang,[a b] Andrei Khlobystov,[c] Vanessa Loczenski Rose,[d] Lee Buttery,[d] Clive J. Roberts[d]

[a]Department of Chemical and Environmental Engineering, Faculty of Engineering, University of Nottingham, University Park, Nottingham NG7 2RD, UK

[b]Promethean Particles Ltd, 6 Faraday Building, Nottingham, NG7 2QP, UK

[c]School of Chemistry, University of Nottingham, University Park, Nottingham, NG7 2RD, UK

[d]School of Pharmacy, Centre for Biomolecular Science, University of Nottingham, University Park, Nottingham, NG7 2RD, UK

Contents

1. Synthesis of Hydroxyapatite Nanoparticles

All reagents were purchased from Sigma Aldrich, UK, and used without any further purification.

For 'standard' synthesis of HA, 0.015 M aqueous ammonium phosphate dibasic, $(NH_4)_2HPO_4$, was pumped (20 ml/min) into a preheater set to 200 ˚C then flowed into the inner tube of the nozzle reactor (downflow). At the same time, 0.05 M calcium nitrate tetrahydrate, $Ca(NO_3)_2.4H_2O$, was pumped (10 ml/min) at ambient temperature into the outer tube (upflow) of the reactor. Subsequent to the mixing point, the product stream is cooled to near-ambient conditions and particles are collect from the outlet of the back pressure regulator (BPR). The sample produced under these conditions was called HA-1. There are several process variables which can be easily altered with this process, including precursor type, precursor concentration, pH, flow rates, flow ratios, mixing temperature and the use of additional surfactants. The effects of some of these parameters were explored. For different samples produced, the precursor pH, concentration, or preheater temperature were altered. Table 1 highlights the experimental details. Adjustments to pH were achieved by dropwise addition of either 25% w/w ammonium hydroxide, NH_4OH, or 1 M sodium hydroxide, NaOH.

For sample HA-Zn, 1 $^{w}/_{w}$% (0.0046 M) zinc nitrate hexahydrate, $Zn(NO_3)_2.6H_2O$, was added to the $Ca(NO_3)_2.4H_2O$ solution prior to reaction using the conditions described for HA-1 synthesis.

Samples were washed by centrifuging, decanting the supernatant and re-suspending particles in deionised water, a process which was repeated a minimum of two times.

2. Characterisation

Samples were collected from the rig as aqueous suspensions. They were washed with DI water and freeze dried (-60 ˚C for 48 hours) for subsequent analysis.

Scanning Electron Microscopy (SEM) - images were obtained by mounting powder samples onto a carbon film; sputter coating with gold and using a FEI Quanta 600 electron microscope with a LaB_6 filament source.

High resolutions TEM (HRTEM) - images were acquired using a JEOL 2100F electron microscope equipped with a field emission electron gun (FEG) and operating at 200 keV. Samples were prepared by suspending in methanol, a few drops loaded onto copper-grid mounted carbon films and air dried.

X-ray Diffraction (XRD) patterns were collected using a Bruker AXS D8 Advance diffractometer with Cu Kα radiation (λ = 0.15406 nm) with a voltage of 40 kV and 35 mA current. Scans were taken over a 2θ range of 20-65˚, employing a step size of 0.04 and scan time of 5 seconds per step. DIFFRAC^{plus} EVA software was used for data analysis.

The **Brunauer-Emmett-Teller (BET)** method was used to calculate surface area data, using a Micromeritics ASAP 2010 and nitrogen adsorption at 77K. The samples were degassed under vacuum at 150^{o}C for 12 hours prior to analysis.

3. Biological Cell Assays

An important part of the validation work for this new approach to nano-hydroxyapatite manufacture has been cell viability assays, to indicate that HA produced in the nozzle reactor exhibit no cytotoxic effects on mouse embryonic stem cells (mESC) and can promote stem cell differentiation into an osteoblast lineage.

3.1 *In Vitro* Cell Proliferation Assay

These assays were used to ascertain if HA produced in the continuous hydrothermal rig exhibit a cytotoxic effect, rather than determine cell response to every sample. Therefore, only nanoparticles from sample HA-1 were used for this study. The sample was sterilised by exposure to UV light. Murine embryonic stem cells (mESC) were maintained in standard culture media and osteogenic culture media at 37˚C, 5% CO_2 at 85% relative humidity. Standard culture media contained DMEM growth media, supplemented with 10% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin 100x solution, 1% L-

Glutamine and 100µM 2-Mercaptoethanol. Osteogenic media contained standard culture media and additional supplements (50 µg/ml L-ascorbic acid, 50 mM β-glycerophosphate) guiding stem cells towards osteoblast differentiation.

To analyze the impact of nanoparticles on cell proliferation and cytotoxicity, a 96 well plate was seeded with cells (7000 cells/well) and treated with nanoparticles of various concentrations (1, 10 or 100 µg/ml). Cell proliferation was monitored at 1, 3 and 6 days of culture using a 96® AQueous One Solution Cell Proliferation Assay (Promega, USA). The assay principle is based on the MTS tetrazolium compound being bioreduced into a coloured formazan product that is soluble in culture media. This conversion is accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolic active cells.

3.2 *In Vitro* Bone Nodule Assay

To study osteogenic differentiation and bone mineralization, cells were cultured in 12 well plates (100,000 cells/well) for 21 days in the presence of nanoparticles at various concentrations (1, 10 and 100 µg/ml). To analyze bone nodule formation cells were fixed in 10% formal saline solution (20 min.) followed by a histological staining with 1% Alizarin red solution (5 min.).The cells were washed three times with distilled water to reduce the background intensity. Mineralized nodules are identified as clusters which stain red-brownish colour.

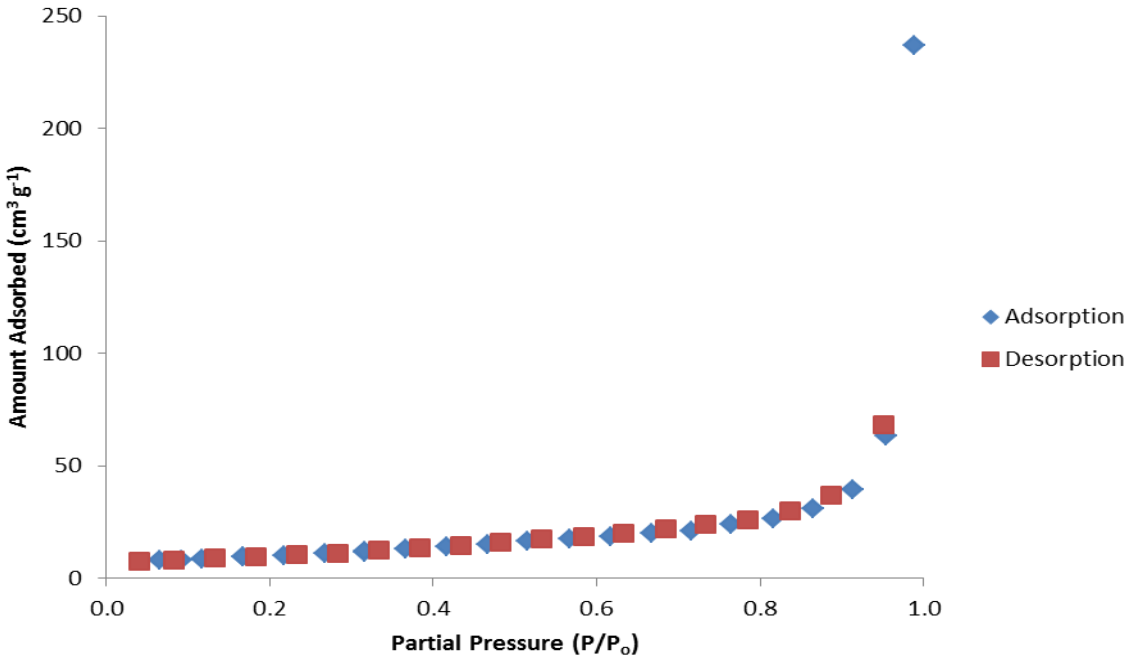

Fig. S1. (a) the adsorption-desorption profiles for HA-5 (C value of 102 and BET surface area of 35m^2 g^{-1} ± 0.8)

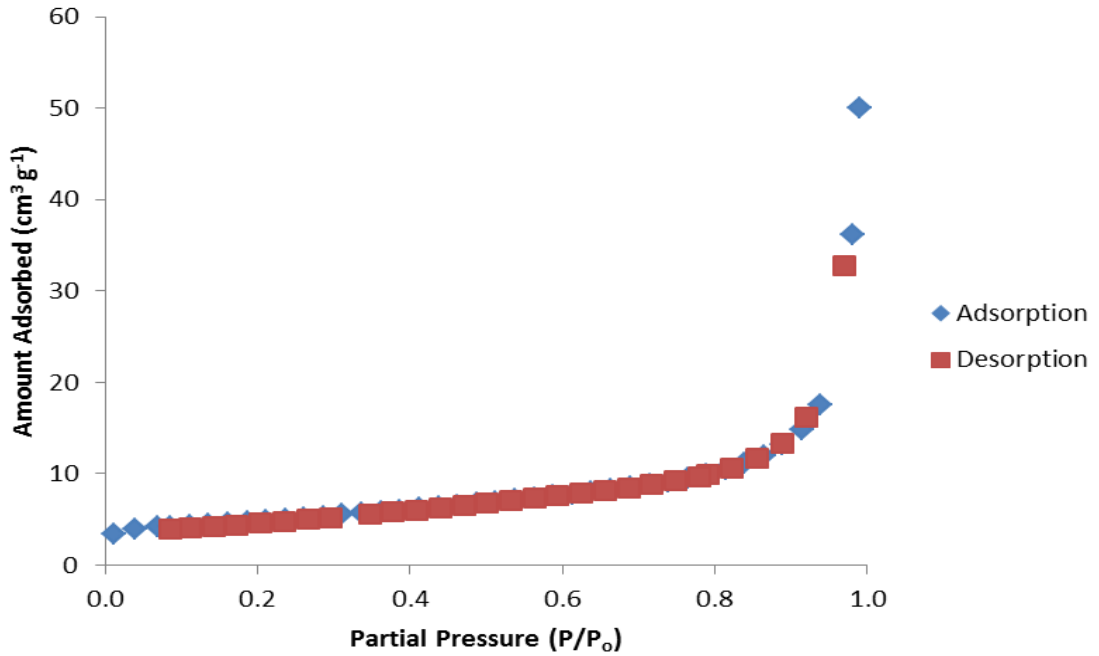

Fig. S1. (b) the adsorption-desorption profiles for HA-1 (C value of 118 and BET surface area of 13.6m^2 g^{-1} ± 0.2)

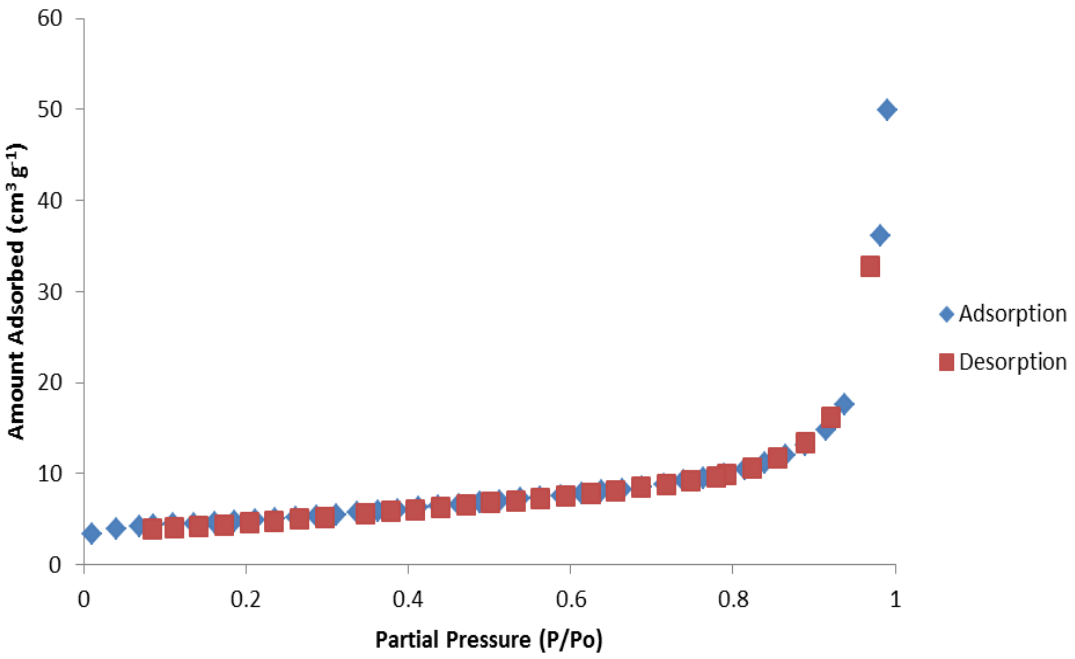

Fig. S1. (c) the adsorption-desorption profiles for HA-4 (C value of 108 and BET surface area of 43m^2 g^{-1} ± 0.2)

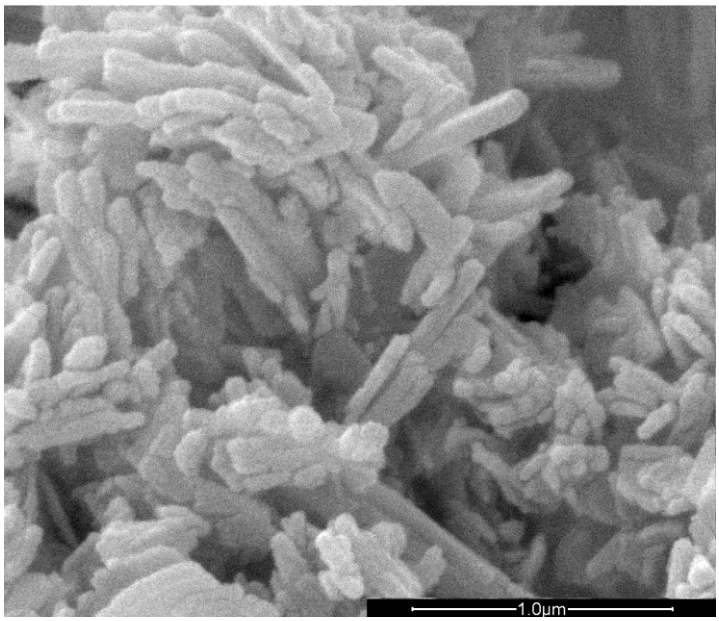

Fig. S2 SEM image of HA-3.

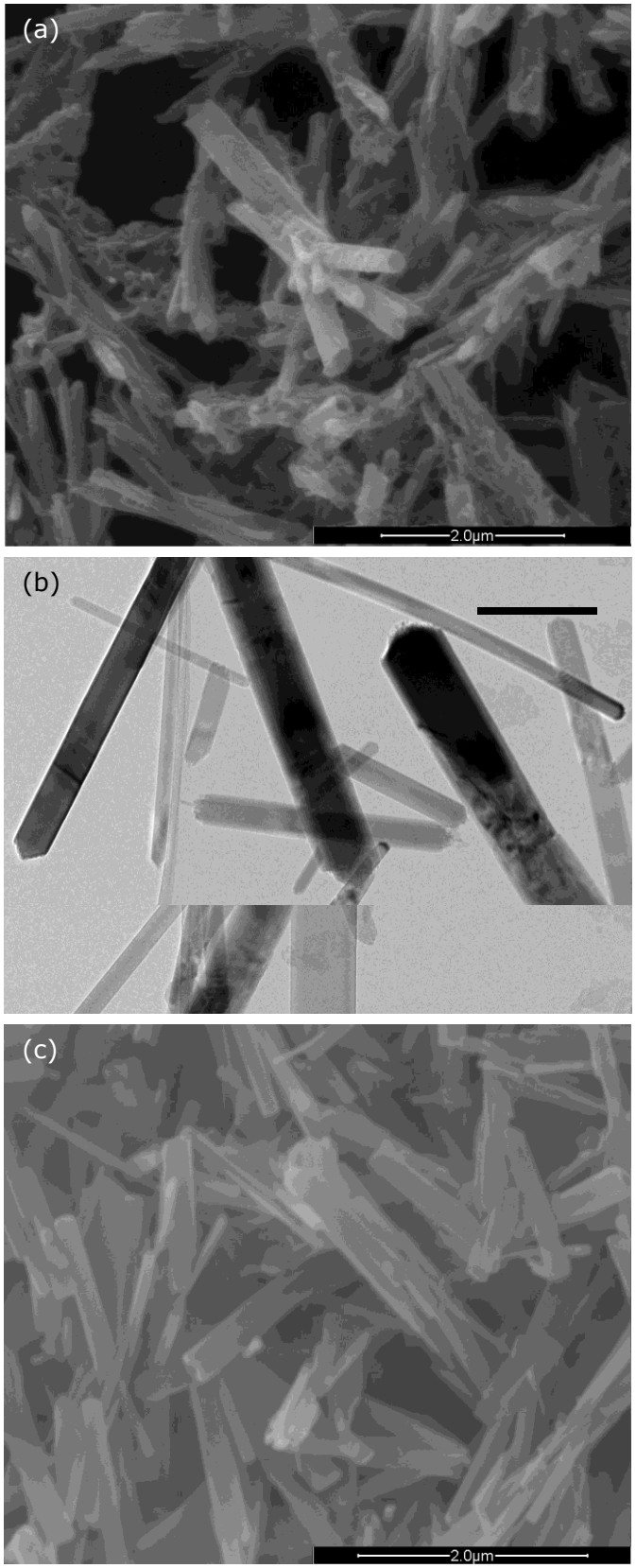

Fig. S3. (a) SEM image of HA-6, (b) TEM image of HA-7, (c) SEM image of HA-8.

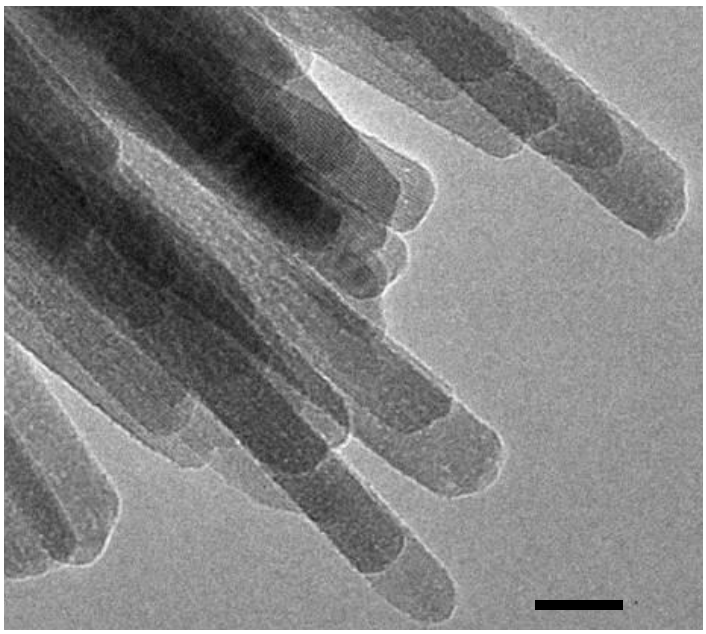

Fig. S4. TEM image of HA-4.

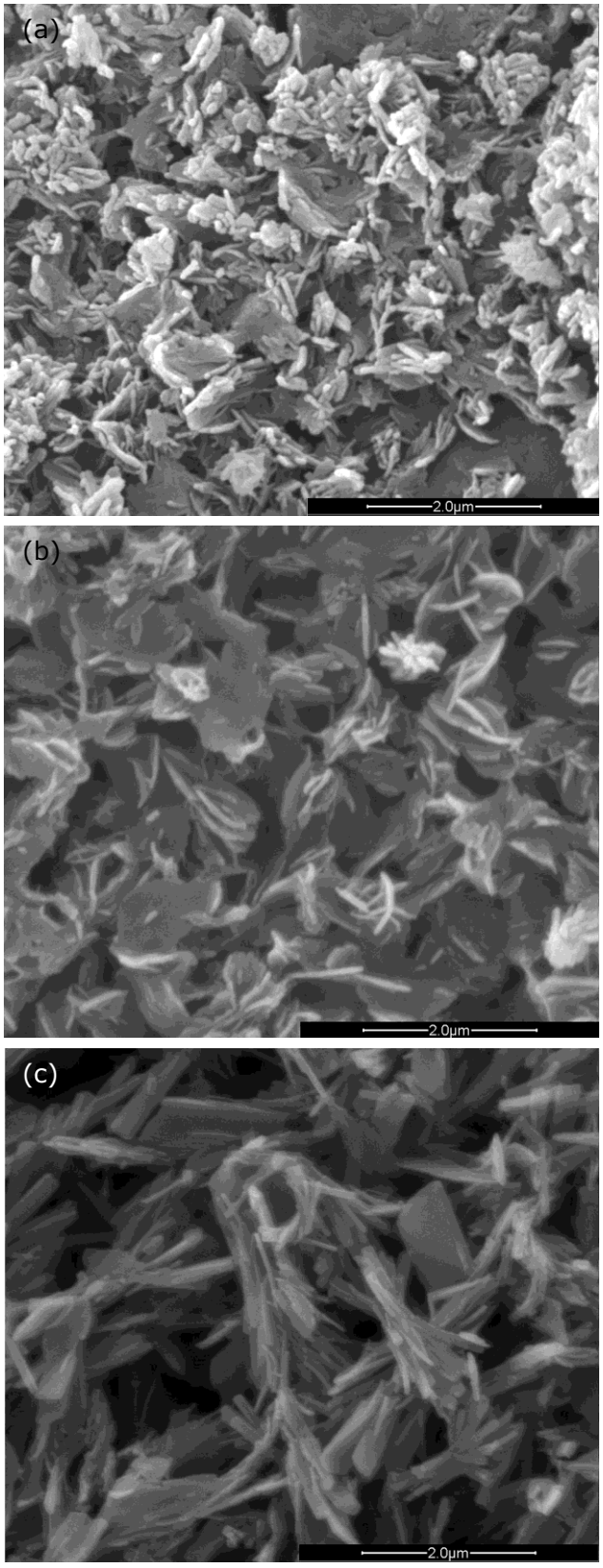

Fig. S5. SEM images of (a) HA-9, XRD data showed this sample to be a mixture of HA and Brushite, (b) HA-10, (c) HA-11.

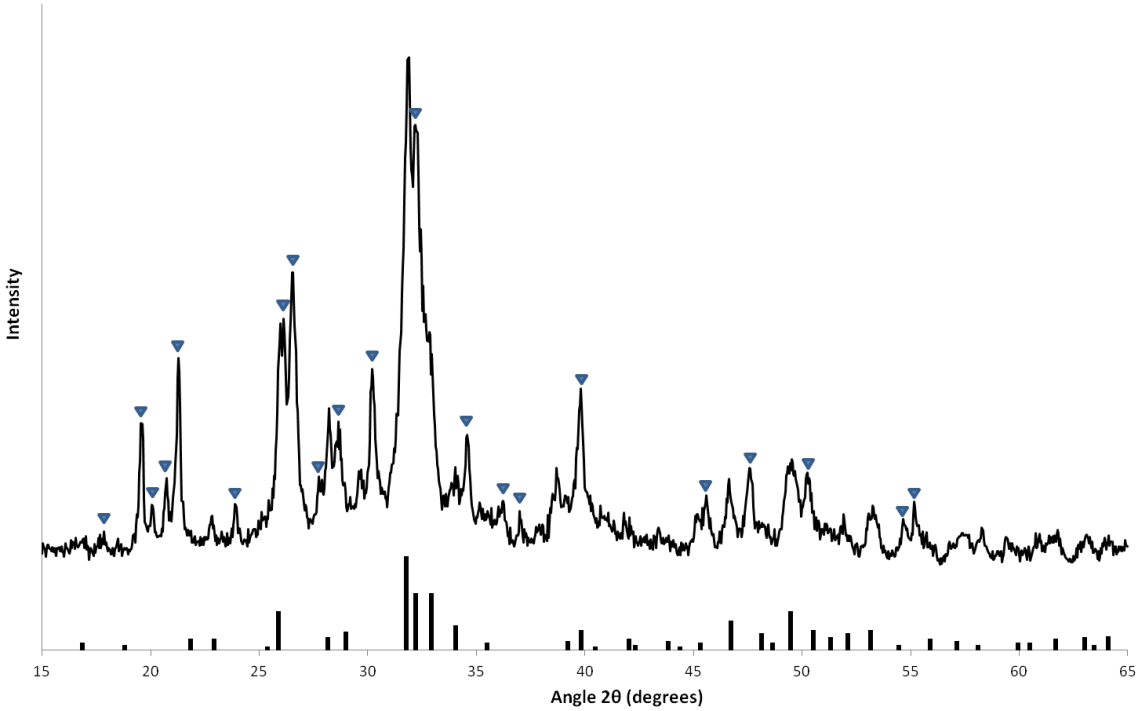

Fig. S6. XRD pattern of HA-Zn; the predominant phases are hexagonal hydroxyapatite (ICCD PDF 74-566, shown at bottom) and monoclinic parascholzite (ICCD PDF 35-495, peaks shown with blue triangles). Additional smaller peaks match calcium hydrogen phosphate (ICCD PDF 77-128).

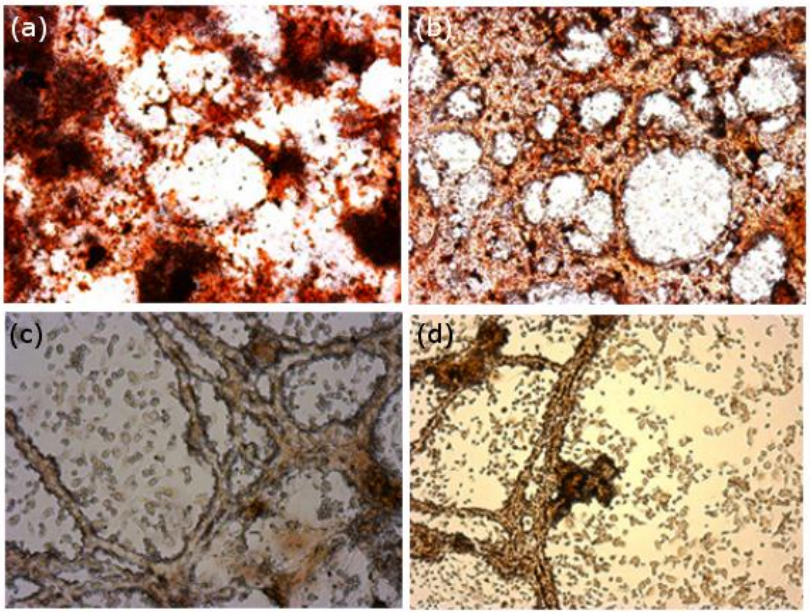

Fig. S7. Images from the bone nodule assay - Cells cultured in osteogenic media with hydroxyapatite from Sample HA-1 at various concentrations, (a) 100 µg/ml; (b) 10 µg/ml; (c) 1 µg/ml; (d) control