Supplementary Methods

Fourier Transform Infrared Spectroscopy (FTIR) analysis

FTIR was used to characterise the chemical bonds present in the CS scaffold during distinct phases in the scaffold treatment process. It was specifically used to identify absorption spectra for the vibrational frequencies of bonds present before and after the porogen removal process. Slices of scaffold were measured using a Bruker Tensor 27 FTIR spectrometer (Brucker UK Ltd, Coventry, UK) with a diamond lens Attenuated Total Reflectance (ATR) module and OPUS software (Opus software Ltd, Grantham, UK). All spectra were recorded in the range 400-4000 cm^{-1} at a resolution of 8 cm^{-1}, scanner velocity of 10 kHz and scan time of 1 min.

EDX

Surface chemical analysis was carried out using a scanning electron microscope (SEM, Philips, XL30) equipped with an Oxford Instruments X-Max -150 EDX Detector. The chitosan and chitosan-nHA surfaces were independently selected and the individual Ca/P ratios were examined using a voltage of 20 kV and a minimum count rate of 2,000 cps.

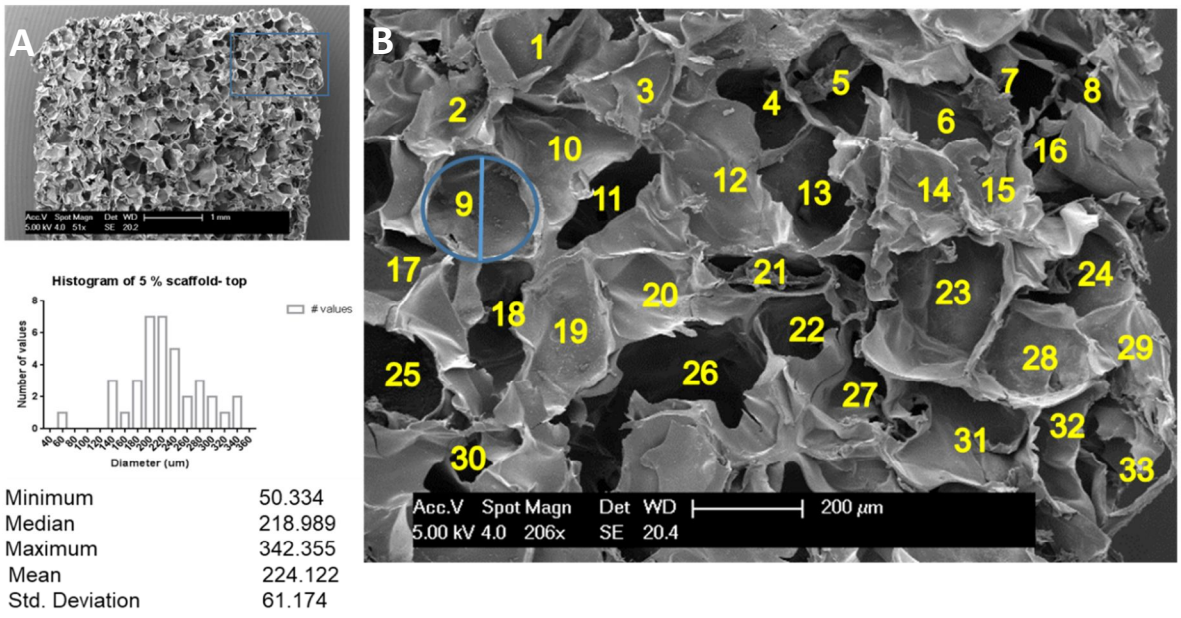
Micro-computed tomography (micro-CT):

Scans were performed using a high-resolution micro X-ray computed tomography system (Skyscan 1174, Bruker) using the following conditions: 50 kV voltage, 800 µA current, voxel resolution of 12 µm. Transmission images were reconstructed using Skyscan supplied software (NRecon) with the resulting two-dimensional image representing a single 12 µm slice (1/256). To distinguish between solid material and porous regions a single threshold value was applied (255/20). Background noise in the images was reduced by removing black and white speckles of less than 100 voxels. Quantitative analysis was obtained using direct morphometry calculations in the Skyscan CTAn software package.

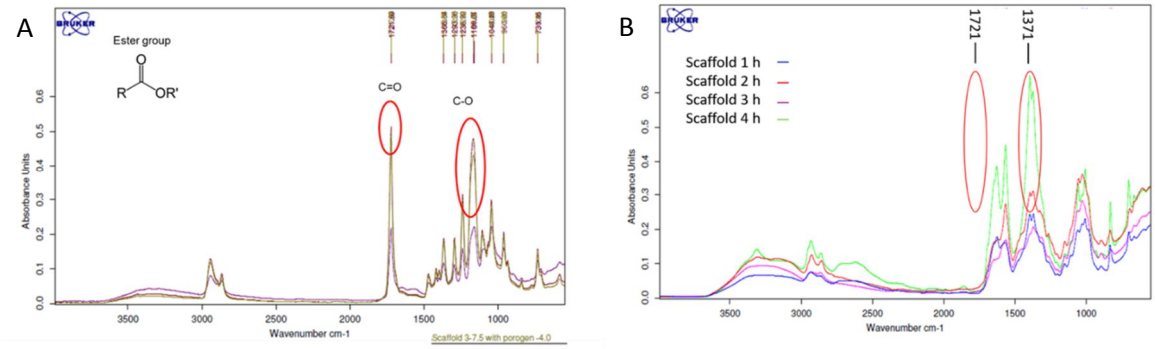
Von Kossa staining

Levels of mineralisation were assessed using a Von Kossa staining method in which a precipitation reaction with silver nitrate occurs in the presence of phosphate ions. A 1% (v/v) solution of silver nitrate (Sigma-Aldrich, Dorset, UK) was prepared in SDW water and added to fixed samples. The scaffolds were exposed to UV light for 20 min and images were taken of the black bone nodules. The scaffolds were washed three times in water and then incubated for 5 min in 2.5 % solution of sodium thiosulphate (Sigma-Aldrich, Dorset, UK) to remove excess silver nitrate.

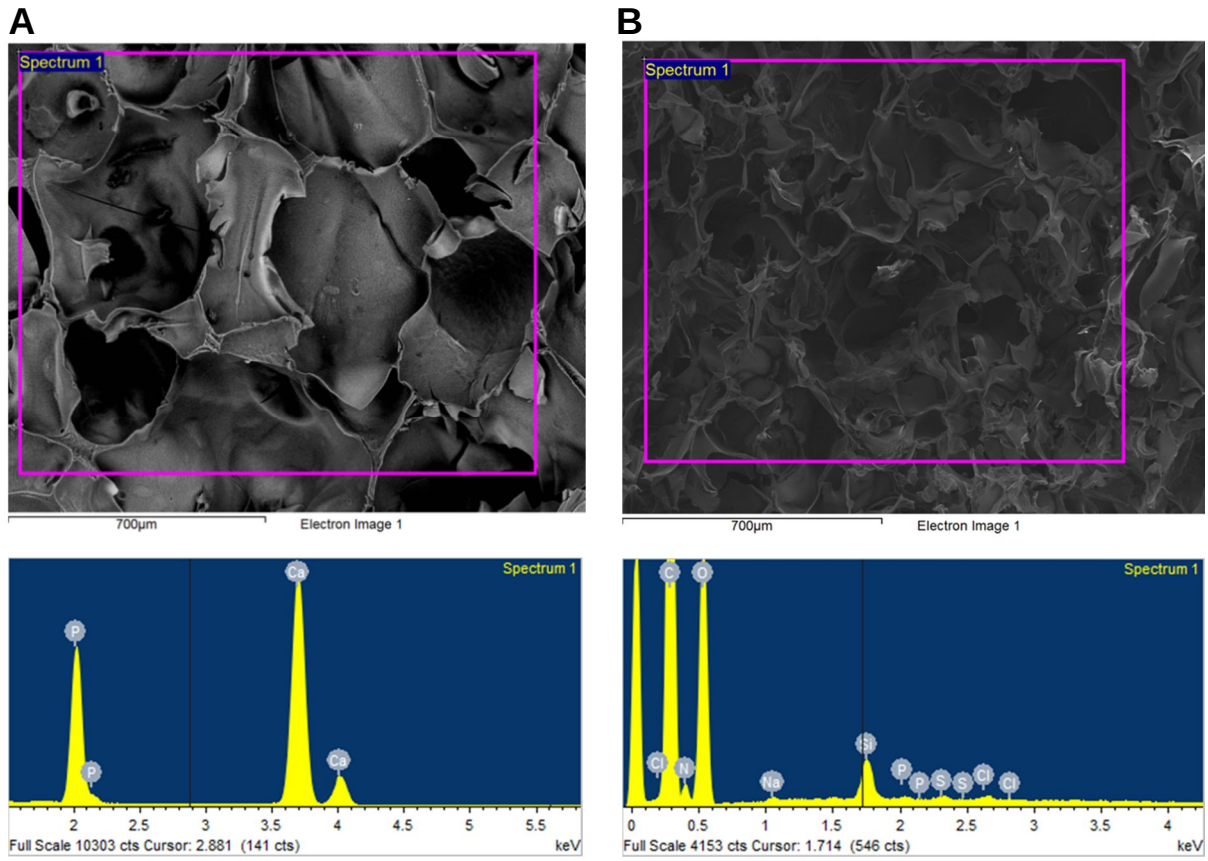
Supplementary Figures



Supplementary Figure 1: SEM image of the internal structure of the chitosan scaffold (A) illustrating the way the pore size was measured (B).



Supplementary Figure 2: FTIR spectra for the chitosan scaffold prior to (A) and after treatment to remove the porogen (B) for 1h, 2h, 3h and 4h, showing absorbance peaks typical of C-O single and double bonds found in esters, indicating the presence of PCL in the scaffold. The spectra show the presence of a carboxyl-salt at absorption values of 1371 cm^{-1} as a result of the treatment with alcoholic potassium hydroxide, and a shift from ester related absorption peaks previously seen at wavelengths of 1720 cm^{-1}.

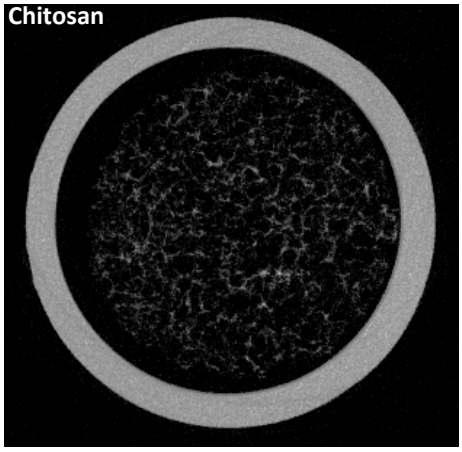


Element	Weight%	Atomic%	Compd%	Formula
P K	16.94	13.35	38.82	P2O5
Ca K	43.72	26.63	61.18	CaO
O	39.33	60.02		
Totals	100.00			

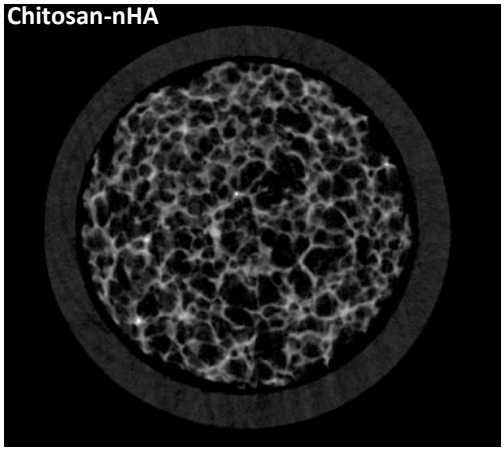
Element	Weight%	Atomic%	Compd%	Formula
P K	43.64	28.57	100.00	P2O5
Ca K	0.00	0.00	0.00	CaO
O	56.36	71.43		
Totals	100.00			

Supplementary Figure 3: SEM/EDX surface analysis of nHA-chitosan (A) and chitosan-only scaffolds (B) and corresponding chemical spectral analysis indicating calcium (Ca) and phosphorus (P) found strictly on the surface areas of chitosan-nHA scaffold with a Ca/P ratio of 1.6.

A



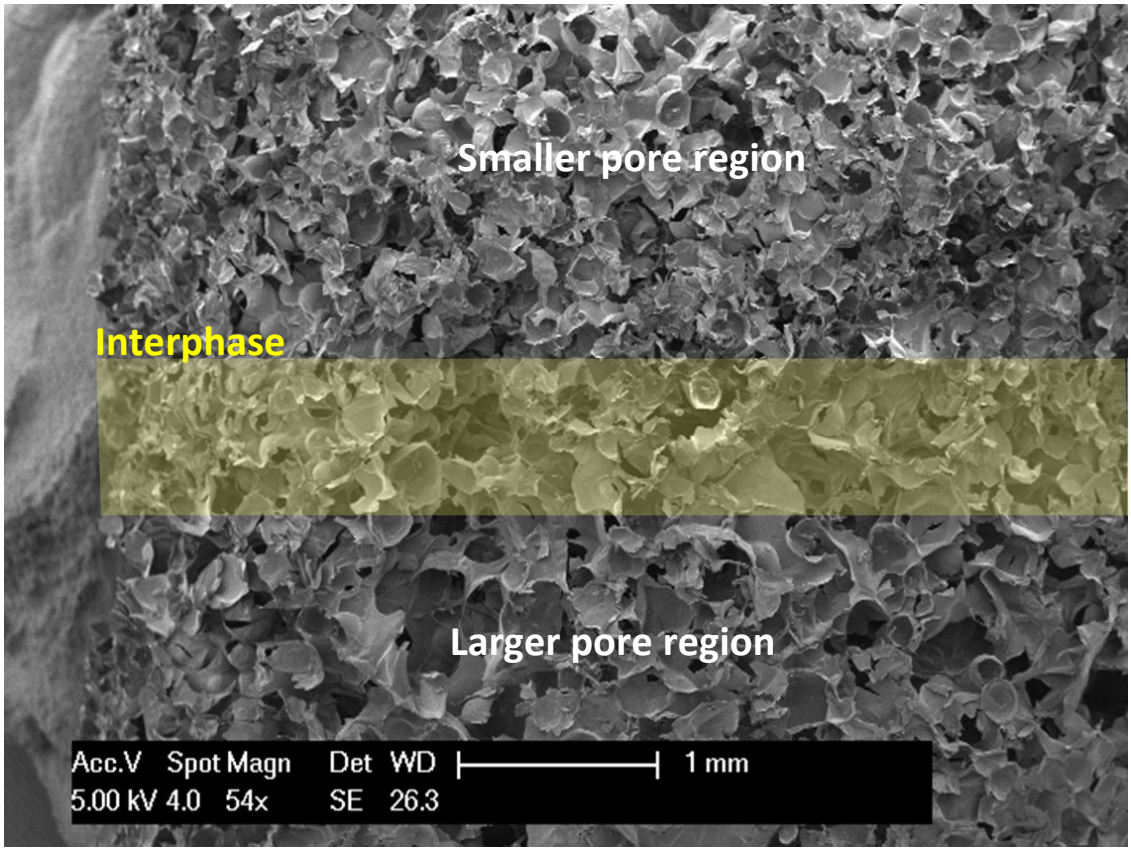
B



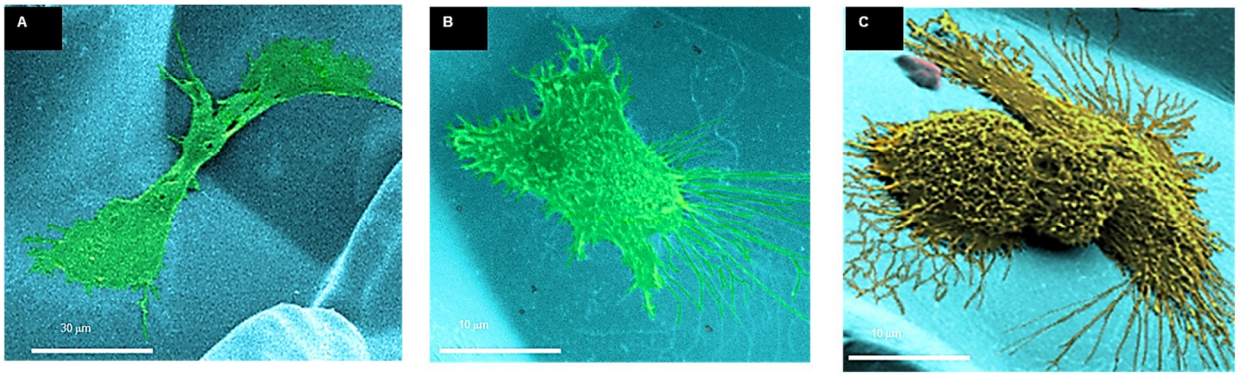
C

	porosity %		pore size (structure separation in μm)	
	large pore	**small pore**	**large pore**	**small pore**
mean	71.75	85.5	300.78	177.48
SD	6.65	6.35	58.64	22.85

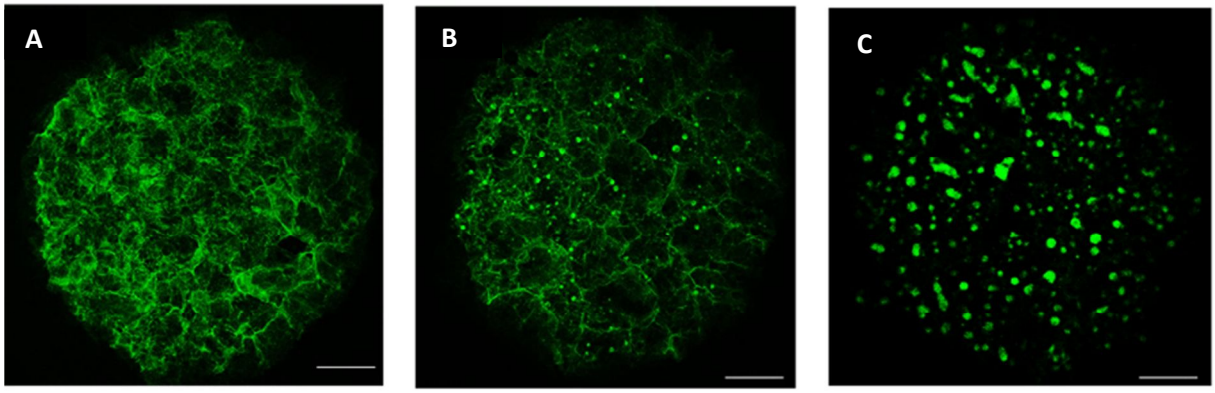
Supplementary Figure 4: microCT images of the chitosan (A) and chitosan-nHA (B) regions of the scaffold, with the resulting porosity and pore size values (C).



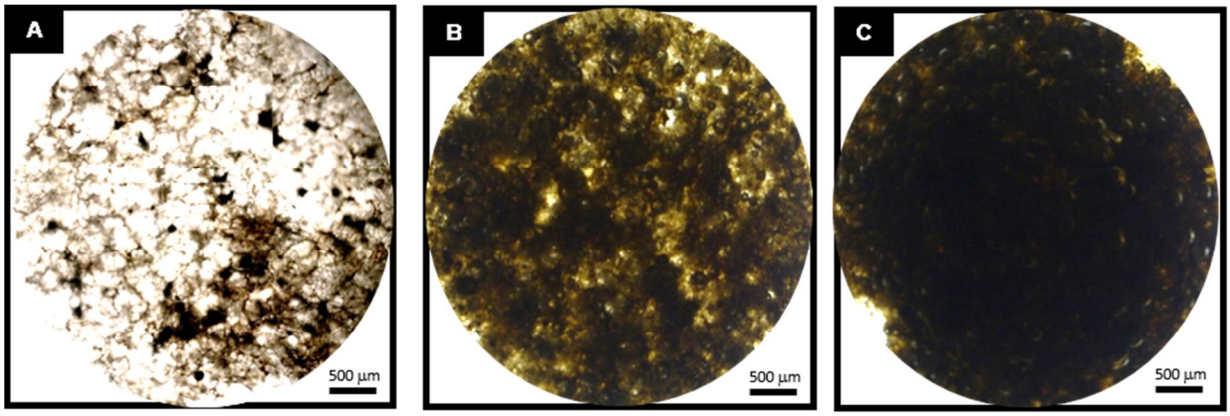
Supplementary Figure 5: SEM image of a cut scaffold illustrating the integrated interphase (yellow) between the two regions.



Supplementary Figure 6: SEM imaging of cell-seeded scaffolds at day 2 post-seeding showing cell adherence and spreading (A,B), and evidence of cell division (C) of MSCs onto chitosan scaffolds, highlighted by artificial colouring. Scale bars: 30 µm (A), 10 µm (B,C).



Supplementary Figure 7: Confocal images showing cells (green) seeded onto chitosan scaffolds cultured for 11 days in standard medium (B) or osteogenic (C) medium, compared to a cell-free scaffold (A) used as control. Scale bar shows 1 mm.



Supplementary Figure 8: Von Kossa staining of cell-seeded scaffolds showing increased mineralisation after 21 days in standard (B) or osteogenic (C) medium, compared to unseeded blank scaffold (A) used as control.