**Supporting Information**

# Resilient and flexible chitosan/silk cryogel incorporated with Ag/Sr co-doped nanoscale hydroxyapatite for osteoinductivity and antibacterial properties

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# 1 Experimental Section

*1.1 Materials*

Dopamine hydrochloride and Polyethylene glycol diglycidyl ether (PEGDE) was purchased from Sigma-Aldrich (USA). Chitosan powder (CS, Deacetylation degree >90% ; Viscosity=50~800 mpa.s) was purchased from Zhejiang Gold·Shell Pharmaceutical Co., Ltd, China. Raw silk was purchased from Shengzhou Xiehe Silk Co. Ltd, China. Calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$), Silver nitrate ($AgNO_3$), Strontium nitrate ($Sr(NO_3)_2$, Ammonium phosphate ($(NH_4)_2HPO_4$), Ammonium Hydroxide ($NH_3 \cdot H_2O$) were purchased from KESHI, Chengdu, China. Bone morphogenetic proein-2 (BMP-2) was purchased from Rebone Biological Technology Ltd. Shanghai, China. Live/dead cell assay kits were purchased from R&D, Ltd., USA. The BCA assay kits and ALP assay kits were purchased from Nan Jing Jian Cheng Ltd., China. Fetal bovine serum (FBS), α-MEM and 1% penicillin-streptomycin solution were purchased from Hyclone, USA.

*1.2 Preparation of ion-doped HA nanoparticles (HA-NPs)*

The HA-NPs were prepared by a hydrothermal method according to a previous report[1]. Firstly, $Ca(NO_3)_2 \cdot 4H_2O$ (9 g) and $(NH_4)_2HPO_4$ (3.02 g) were separately dissolved in distilled water (50 mL) to form two aqueous solution. Secondly, under condition of vigorous stirring, the 50 mL of $(NH_4)_2HPO_4$ solution was added into the 50 mL of $Ca(NO_3)_2$ solution which maintained a Ca/P molar ratio as 1.67. Meanwhile, an $NH_4OH$ solution was added immediately to adjust the pH value of the mixed solution to 10. After vigorous stirring for 1 hour, the suspension was hydrothermally treated at

160 °C for 12 h. Next, the precipitates were washed with the distilled water and separated by centrifugation three times. Finally, the nano-sized HA slurry was freeze-dried to give the HA-NPs.

Previous studies demonstrated that only small doses of Ag could be doped into the HA lattice because the maximum amount of Ag substitution was very low[2]. Nevertheless, the low Ag content already has excellent antibacterial properties. In contrast, Sr could replace Ca sites without restriction. However, the excessive Sr was harmful to bone regeneration and only a suitable amount of doped Sr could improve osteoconductivity of HA[2, 3]. According to these studies, we designed the amount of Ag and Sr that was doped into HA. In our studies, the Ag/(Ca+Ag) molar ratio is equal to 1 : 20 in the AgHA-NPs; the Sr/(Ca+Sr) molar ratio is equal to 1 : 10 in the SrHA-NPs; the Ag/Sr/(Ag+Sr+Ca) molar ratio is equal to 1 : 2 : 20 in the AgSrHA-NPs. The reactants of the raw material for the synthesis of Ag and Sr doped HA-NPs are listed in **Table S1**. All the other synthetic conditions and procedures were the same as those for the HA-NPs.

*1.3 Preparation of SF solution*

SF solution was prepared according to a method reported previously [4]. Firstly, silk cocoons (10 g) were cut into pieces and degummed (to remove sericin) with a boiling in $Na_2CO_3$ 0.025 mol $L^{-1}$ solution (1 L) for 30 min, Secondly, the silk fibers were washed with deionized water and dried at 37 °C. Purified SF fibers were dissolved in 9.3 M LiBr and then dialyzed against water using cellulose dialysis membrane for 72

h. Dialysis was carried out to remove LiBr from the silk fibroin solution with frequent change of water on regular time interval. The final concentration of SF solution to be used was 2 wt. % and was determined gravimetrically by drying the solution.

*1.4 Model building and DFT Calculation*

The CASTEP module is an ab initio program which allows ground state energies to be calculated and the unit cell to be optimized using a density functional theory [5]. The ab initio method can produce credible total energies and atomic forces, which can be used to acquire the balanced arrangement of atoms [6].

All the structural optimization calculations used the generalized gradient approximation (GGA) of the PBE exchange–correlation[7]. The electronic ground state was reached using a conjugate gradients algorithm, and the BFGS optimization method was used to relax the atomic structure. The cut off energy of the plane waves was set at 380 eV. Brillouin zone samplings were performed using the Monkhost-Pack scheme with a 4×4×4 k-point grid. The convergence criteria for the geometry optimization and energy calculations were set to (a) a self-consistent field tolerance of $10^{-6}$ eV/atom; (b) an energy tolerance of $10^{-5}$ eV/atom; (c) a maximum force tolerance of 0.3 eV/nm; (d) a maximum stress tolerance of 0.05 GPa; (e) a maximum displacement tolerance of $10^{-4}$ nm. The cell sizes and the atomic positions were loosened without any constraints in the process of the DFT calculation.

The formation energies of different substitution models were calculated using the DFT method in order to obtain the most stable doped HA configurations. The formation energy was given by the equation: $E_f = E_{doped-HA} - E_{HA} + \sum_1^n n_i\mu_i$, where $E_{HA}$ and $E_{doped-HA}$ are energies of perfect HA and doped-HA, respectively, $n_i$ denotes the numbers of the corresponding ionic species to be removed from ($n_i$>0) or added ($n_i$<0) to the HA unit cell and $\mu_i$ denotes the chemical potential for an ionic species [8]. In our study, the chemical potentials are approximated by their 0 K energies per atom or molecule and their values are estimated using various chemical sources and sinks [9].

*1.5 Porosity and water absorption ability*

The porosity of cryogels was measured by the following step, reference to previous literature[10]. First, the volume (V) and weight ($W_1$) of various cryogels (Φ 10 × 1 $mm^2$), including CS/SF, HA/CS/SF, AgHA/CS/SF, SrHA/CS/SF and AgSrHA/CS/SF, were measured. Then, all the cryogels were immersed into ethanol solution (100%) at room temperature for 10 min. After absorption of alcohol, the weight ($W_2$) of various cryogels with alcohol was measured again, respectively. Final, the porosity of various cryogels was calculated as following formula.

Porosity (%) = $(W_1-W_2)/\rho V\times100\%$

The ρ represents the density of alcohol.

The water absorption ability of cryogels was measured by the following step, reference to previous literature[11]. First, the volume (V) and weight (w) of various cryogels (Φ 10 × 1 $mm^2$), including CS/SF, HA/CS/SF, AgHA/CS/SF, SrHA/CS/SF and AgSrHA/CS/SF, were measured. Then, all the cryogels were immersed into distilled water at 37 ℃ for 10 min. After absorption of distilled water, the quality (W) of various cryogels with distilled water was measured again, respectively. Final, the water absorption ability of various cryogels was calculated as following formula.

Water absorption ability = $(W-W_1)/W_1$

*1.6 Antibacterial test in vitro*

The antibacterial test was conducted to evaluate the antibacterial properties of these various cryogels (blank (tissue culture plate with sample), CS/SF, AgHA/CS/SF, SrHA/CS/SF, AgSrHA/CS/SF) against Staphylococcus epidermidis (S. epidermidis, Gram positive bacteria, ATCC 12228) and Escherichia coli (E. coli, Gram negative bacteria, ATCC8739). Each group has four parallel samples. Firstly, 200μL of bacterial suspension ($10^6$ CFU $mL^{-1}$) was cultured with the samples (Φ 10×1 mm) for 12 h. Secondly, Luria-Beriani broth (800 μL) was added into each sample for 12 h continuous culture. Finally, the OD of the bacterial suspension of each sample was measured at 600 nm in the microplate reader.

The antibacterial ratio of cryogels were calculated from the following equation:

$$Antibacterial\ ratio\ (\%) = (OD_{Blank}-OD_{Sample})/OD_{Blank} \times 100\%$$

*1.7 Cell experiment in vitro*

Bone marrow stromal cells (BMSCs) were cultured on various cryogels (Φ 10 × 1 $mm^2$), including HA/CS/SF cryogel, AgHA/CS/SF cryogel, SrHA/CS/SF cryogel, AgSrHA/CS/SF cryogel and BMP-2 incorporated AgSrHA/CS/SF cryogel to evaluate their cytocompatibility. The experiments were performed in accordance with protocols approved by the local ethical committee and laboratory animal administration rules of China. Firstly, BMSCs were obtained from 7-days-old Sprague-Dawley (SD) rats as described in previous papers[12]. BMSCs were seed on the various cryogels with a density of $5\times10^4$ cells/sample and cultured in α-MEM supplemented with 10% FBS and 1% penicillin-streptomycin solution at 37 °C in a 5% $CO_2$ incubator.

For cell attachment study, the BMSCs on various cryogels were washed twice with PBS, and then fixed with 2.5% glutaraldehyde for 4 h at room temperature after 3 days of incubation. The cells were then subjected to step dehydration with a graded series of ethanol/water solutions (30%, 70%, 90%, and 100%) for 10 min each step. Finally, the cells were dried by critical point drying and gold-sputtered prior to SEM observation. The morphology of the BMSCs was assessed using fluorescence staining. Firstly, after 7 days in culture, the medium was removed from the wells and the cells on various cryogels were washed twice with PBS. Secondly, the cells were stained using the Live/dead cell assay kits for 2 h. Thirdly, the stained samples were washed twice with PBS and were observed under a fluorescent microscope (DMIL, Leica,

Germany).

Cell proliferation was evaluated by the MTT assay after 3 and 7 days of culture. First, the culture medium in the 48-well plate was replaced with MTT solution (0.5 mg/mL, 0.2 mL of α-MEM with 10% FBS). After 4 h incubation, the Dimethyl Sulfoxide (DMSO) was used to replace the MTT formazan and dissolve the MTT formazan at 37 °C for 15 min. Final, 100 uL reagents were carefully transferred to 96-well plates. The optical density was measured at 570 nm using a microplate reader.

Cell differentiation was evaluated by the alkaline phosphatase activity (ALP) assay. After 14 days of culture, the medium was removed from the wells. Subsequently, the cells on various cryogels were washed twice with PBS and lysed with the Triton X-100 (400 μL, 1.0% v/v). The lysate was centrifuged, and the supernatant was employed for ALP activity determination. The final ALP activity was normalized with respect to the total protein content obtained from the same cell lysate. The total protein concentration of the cell lysate of each sample was measured using the BCA Kit. ALP activity was measured using the ALP Assay Kit. In each case, four specimens were tested, and the assay was repeated three times.

*1.8 Animals experiment in vivo*

All surgical procedures were performed in accordance with protocols approved by the local ethical committee and laboratory animal administration rules of China. Four

groups of implants materials (Φ 5 × 1 $mm^2$), including blanks groups, HA/CS/SF cryogel, AgSrHA/CS/SF cryogel, BMP-2 loaded AgSrHA/CS/SF) cryogel were also implanted into the critically sized cranial defect sites in four SD rats to evaluate the osteoinductivity of these cryogels. Sixteen 2-month-old male SD rats, weighing 300~400 g, were purchased from Chengdu Dossy Biological Technology Co., Ltd. The surgical procedure was performed under general sterile conditions. Firstly, a 10-mm defect was made at the skull. Secondly, the critically sized cranial defect 5 mm in diameter was made by a dental drill. Finally, the samples were implants into the critically sized cranial defect and the wound was carefully sutured. All rats were administered with penicillin for 3 days. After 12 weeks of implantation, the cryogels were harvested for hematoxylin and eosin (H&E) and Masson's trichrome staining. All histological staining was observed by a light microscope (Olympus BX63, Japan). The new bone area ratio (NBAR) was defined as follow. The selected area (SA) with 5 mm long and 0.7 mm wide was determined around the bone defect of H&E stained section. Then, the new bone area (NBA) was measure. The NBAR of all groups were calculated according to the following equation.

$$NBAR = \frac{NBA}{SA} \times 100\%$$

*1.9 Statistical Analysis.*

The significance of differences ($p \leq 0.05$) between the test groups were analyzed using the one-way analysis of variance (ANOVA) followed by Tukey multiple comparison post hoc test.

## 2 Results

**Table S1.** The ingredient of raw material for synthesis of Ag and Sr doped HA-NPs

| **Materials** | HA | AgHA | SrHA | AgSrHA |
|---|---|---|---|---|
| $Ca_2(NO_3)_2 \cdot 4H_2O$ | 9 g | 8.55 g | 8.1 g | 7.65 g |
| $(NH4)_2HPO_4$ | 3.02 g | 3.02 g | 3.02 g | 3.02 g |
| $AgNO_3$ | 0 g | 0.324 g | 0 g | 0.324 g |
| $Sr(NO_3)_2$ | 0 g | 0 g | 0.807 g | 0.807 g |
| $H_2O$ | 100 mL | 100 mL | 100 mL | 100 mL |
| $NH_3 \cdot H_2O$ | 50 mL | 50 mL | 50 mL | 50 mL |

**Table S2.** The ingredient of various Ag and Sr doped HA/CS/SF was described.

| **Materials** | CS/SF | HA/CS/SF | AgHA/CS/SF | SrHA/CS/SF | AgSrHA/CS/SF |
|---|---|---|---|---|---|
| CS | 0.4 g | 0.4 g | 0.4 g | 0.4 g | 0.4 g |
| SF | 0.2 g | 0.2 g | 0.2 g | 0.2 g | 0.2 g |
| $H_2O$ | 20 mL | 20 mL | 20 mL | 20 mL | 20 mL |
| acetic acid | 400 μL | 400 μL | 400 μL | 400 μL | 400 μL |
| HA-NPs | 0 g | 0.6 g | 0 g | 0 g | 0 g |
| AgHA-NPs | 0 g | 0 g | 0.6 g | 0 g | 0 g |
| SrHA-NPs | 0 g | 0 g | 0 g | 0.6 g | 0 g |
| AgSrHA-NPs | 0 g | 0 g | 0 g | 0 g | 0.6 g |

**Table S3.** The porosity of various composite cryogels.

| Samples | Porosity (%) |
|---|---|
| CS/SF cryogel | 90.2±3.6 |
| HA/CS/SF cryogel | 85.3±2.3 |
| AgHA/CS/SF cryogel | 82.3±4.1 |
| SrHA/CS/SF cryogel | 84.6±1.8 |
| AgSrHA/CS/SF cryogel | 83.9±2.4 |

**Table S4.** The water absorption of various composite cryogels (1 $cm^3$).

| Samples | Water absorption ability |
|---|---|
| CS/SF cryogel | 32.5±4.5 |
| HA/CS/SF cryogel | 15.8±2.6 |
| AgHA/CS/SF cryogel | 16.4±3.1 |
| SrHA/CS/SF cryogel | 15.9±2.1 |
| AgSrHA/CS/SF cryogel | 15.2±3.2 |

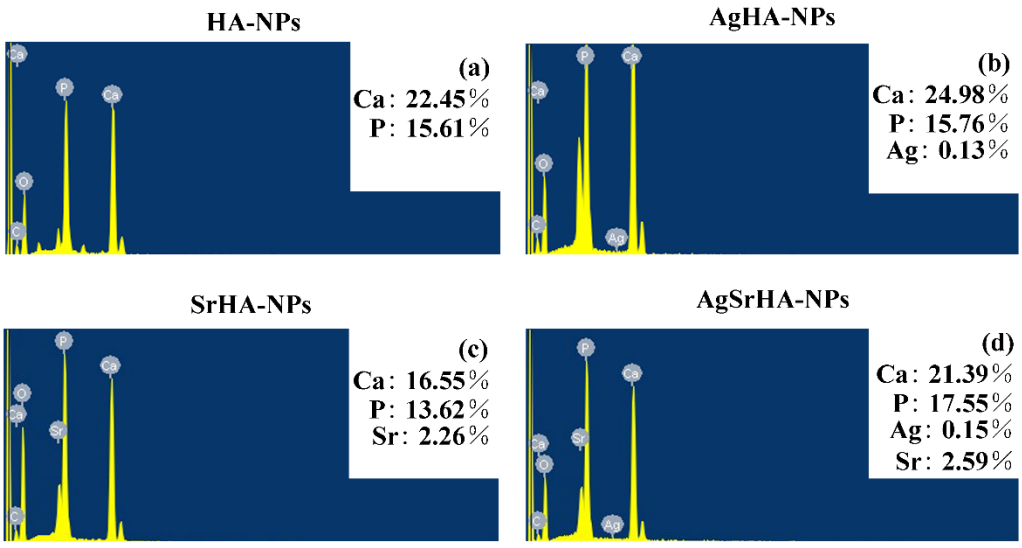


**Figure S1.** The EDS spectra of the various Ag and Sr doped HA-NPs.

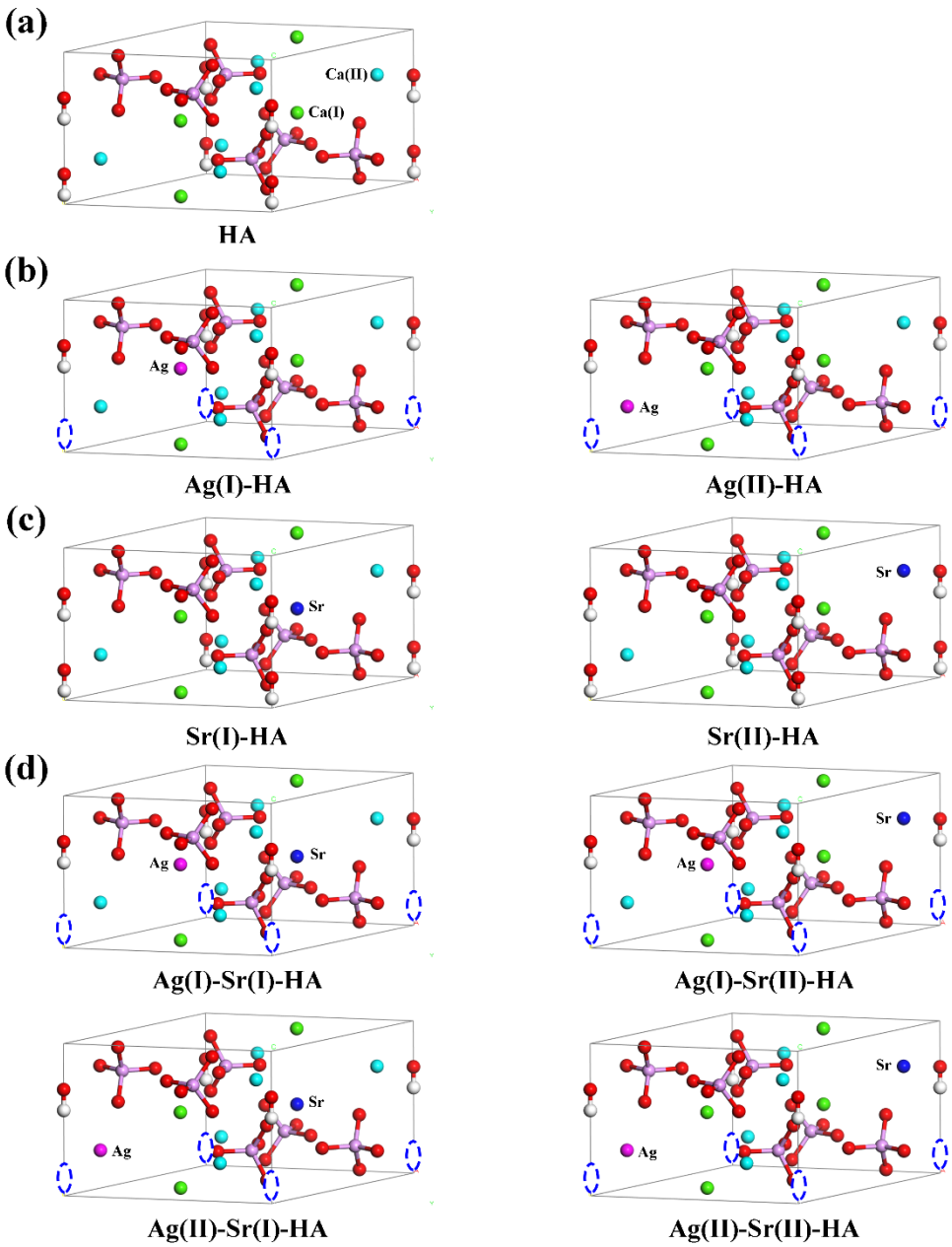


**Figure S2.** Nine ion-doped HA models of different doping sites of Sr and Ag.

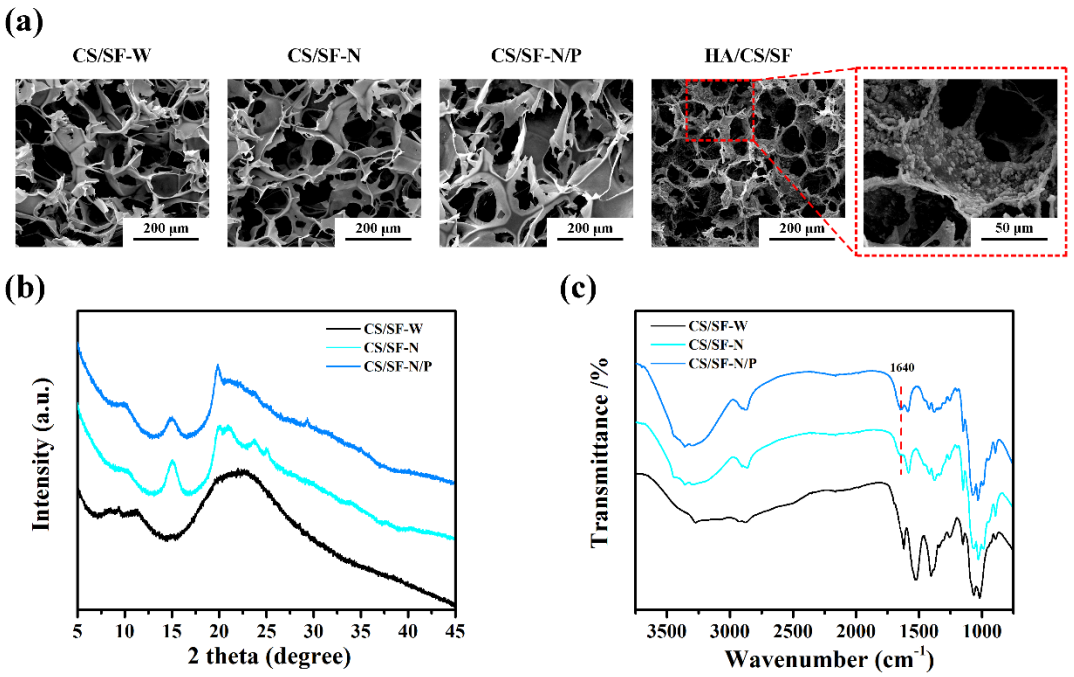


**Figure S3.** The SEM micrographs (a), XRD (b) and FT-IR (c) of various cryogels. The CS/SF-W, CS/SF-N and CS/SF-N/P indicate untreated CS/SF cryogels, NaOH treated CS/SF cryogels and NaOH/PEGDE treated CS/SF cryogel, respectively.

The HA/CS/SF and CS/SF cryogels showed microporous sponge-like structure which was similar to a bone tissue structure. And the structure was conducive to nutrients entry and bone tissue ingrowth. After HA-NPs incorporation, the interconnected 3D porous structures of the CS/SF cryogel was retained, and the HA-NPs were uniformly embedded in the struts of the pores. The XRD patterns (Figure S3b) of the various cryogels showed that the CS/SF generated an intense diffraction peak at 12°~18° after an alkali treatment, which demonstrated that the alkali treatment can induce the formation of partial crystallized CS. The FT-IR spectra of various cryogels were

shown in Figure S3c. Compared with CS/SF-N cryogel, the CS/SF-N/P cryogel exhibited the absorption peak of (-NH-) imino groups at 1640 $cm^{-1}$, which were ascribed to the reacting epoxy groups of PEGDE with amino groups of CS and SF composite network.


Video S1.jpg

**Video S1.** The squeezed and folded HA/CS/SF cryogel with a "4" shape was able to recover to its initial shapes immediately after being immersed into water.

## 3 References


1. S. P. Pathi, D. D. W. Lin, J. R. Dorvee, L. A. Estroff and C. Fischbach, *Biomaterials*, 2011, **32**, 5112-5122.
2. M. Šupová, *Ceram. Int.,* 2015, **41**, 9203-9231.
3. Z. Jacek, N. Michael, B. Michael, S. Andrea and S. A. M. Tofail, *Phys. Chem. Chem. Phys.*, 2012, **14**, 3435-3443.
4. Y. Wang, D. J. Blasioli, H.-J. Kim, H. S. Kim and D. L. Kaplan, *Biomaterials*, 2006, **27**, 4434-4442.
5. K. Refson, *CCLRC Rutherford Appleton Laboratory*, 2004.
6. F. Ren, Y. Leng, R. Xin and X. Ge, *Acta Biomater.,* 2010, **6**, 2787-2796.
7. J. P. Perdew, J. Chevary, S. Vosko, K. A. Jackson, M. R. Pederson, D. Singh and C. Fiolhais, *Phys. Rev. B.,* 1993, **48**, 4978.
8. F. Ren, X. Lu and Y. Leng, *J. Mech. Behav. Biomed. Mater.,* 2013, **26**, 59-67.
9. H. Chappell and P. Bristowe, *J. Mater. Sci.: Mater. Med.,* 2007, **18**, 829-837.
10. V. Karageorgiou and D. Kaplan, *Biomaterials*, 2005, **26**, 5474-5491.
11. G. H. Yew, A. M. M. Yusof, Z. A. M. Ishak and U. S. Ishiaku, *Polym. Degrad. Stab.* 2005, **90**, 488-500.
12. Z. Wang, C. Li, J. Xu, K. Wang, X. Lu, H. Zhang, S. Qu, G. Zhen and F. Ren, *Chem. Mater.*, 2015, **27**, 848−856.