Structure and properties of various hybrids fabricated by silk nanofibrils and nanohydroxyapatite†

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To harvest silk fibroin (SF) based organic/inorganic composites with various general properties (e.g. hard or soft), the strategies of vacuum filtration and centrifugation were employed in this work to produce a film and hydrogel of SF-nanofibril/nanohydroxyapatite, respectively. It was found that the SF-nanofibril mediated the mineralization of hydroxyapatites (HAP) in situ and the morphology of such organic/inorganic nanohybrids presented a "flower-like" structure, mainly because of the strong interaction between SF-nanofibrils and nanohydroxyapatites. On the other hand, the extracellular matrix (ECM) like SF/HAP hydrogel illustrated not only an adequate mechanical strength, but also a remarkable thixotropy, with the storage modulus (G') being able to recover to 85% within 50 seconds when a large shearing strain (5000%) was applied. Moreover, the mechanical properties of these well-organized materials were adjustable for varied demands, and the whole fabrication process was simple and eco-friendly. Therefore, all results indicate that hybrids of SF-nanofibril/nanohydroxyapatite have promise in applications, particularly in bone tissue engineering.

Introduction

Millions of patients are suffering from organ failure or tissue loss because of diseases and trauma, and tissue engineering is proving to be the most promising way to overcome this challenge. As one of the key factors in tissue engineering, biomaterials that are responsible for supporting cell growth, differentiation, guiding the formation of an extracellular matrix (ECM) and tissue regeneration are always being focused on, both for aspects of their bioactivities and for their mechanical behaviours.1–3

Biomineralization is a widely-used approach to develop mineral crystals with controllable morphologies, polymorphs and unique properties under moderate and eco-friendly conditions.4–7 Bone self-repairing is the most complex biomineralization process in nature in terms of its dynamic process and complicated structure. Bone is a vascularized tissue which contains 70% inorganic material (most of this is nano-scaled hydroxyapatite crystals) and 30% of organics, such as collagen, proteoglycans, glyco-proteins and sialoproteins, by dry weight.8 Also, bone has various well-organized structures at a number of length scales which work jointly to carry out different chemical, mechanical and biological functions.9,10

In past decades, researchers have attempted to reproduce a bone-like complex hierarchical structure to get novel materials for tissue engineering, bone grafting or other medical applications.11,12 Among these methods, self-assembled structures based on collagen have been produced with the purpose of regulating the mineralization of hydroxyapatite (HAP) “in situ”. In addition, many non-collagenous nanofibrous scaffolds, including both natural and synthetic polymers, such as silk fibroin, chitosan, gelatin, polycaprolactone, poly(l-lactic acid), and poly(glycolic acid) as well as their combinations, have also been applied as matrices.13–18

Ideal materials for bone tissue engineering are matrices that are able to mimic both chemical composition and physical structure. Also, these materials should have some biological functions like the native extracellular matrix (ECM), such as acting as a temporary substrate allowing cell growth and tissue development. At the same time, biocompatible scaffolds used for bone tissue engineering should be able to provide appropriate mechanical support and exhibit favorable surface properties, such as promoting adhesion, proliferation and differentiation of cells.19

The chemical composition of HAP is similar to that of the inorganic component of bone matrix. Chemical bonding with...
the host tissue helps HAP in clinical applications compared to most other bone substitutes, such as allografts or metallic implants.26 The other advantage of HAP is its biocompatibility, slow biodegradability in situ, and good osteoconductive and osteoinductive capabilities.21 Nanocrystalline HAP displays improved enhanced densification due to its greater surface area, which may improve fracture toughness, as well as other mechanical properties.22

However, it is known that in situ HAP mineralization within an ECM is a more than complicated process to imitate, so the resultant composites are normally heterogeneous.23 Therefore, growing attention has recently centred on the extensive control over molecular recognition and assembly as well as the macroscopic order of the HAP and ECM-like hybrids.19–22 At the same time, finding a balance between fabricating large-scale hybrid materials and controllable size and morphology is quite important.

Bombyx mori silk fibroin (SF) is a widely used natural macromolecule with substrate recognition properties and a self-assembly tendency. Its molecule contains over 5000 amino acid residues which primarily comprise glycine (G), alanine (A), and serine (S). These three amino acid residues compose the repetitive GAGAGS motif, which could fold into the anti-parallel β-sheet structure in the crystalline regions of natural silk fibers.23,24 Silk fibroin can be processed to produce a variety of new materials with different properties from an aqueous solution to a hydrogel or film, with a porosity dependent upon protein concentration and gelation temperature.24 In terms of cell compatibility, degummed silks are arguably well suited for cell culture purposes. SF coated membranes (prepared using aqueous fibroin solutions) allowed better adherence and consequently greater proliferation of fibroblasts compared to non-coated ones.25 Silk fibroin films have also been shown to be biocompatible with human mesenchymal stem cells in vitro, and even enhanced cell proliferation in comparison to collagen scaffolds. In vivo, inflammatory reactions to the silk films were either similar to or less than those to collagen.26 Our recent studies have found that the morphs and morphologies of some mineral crystals, especially aragonite, calcite and vaterite, can be mediated by the β-sheet of SF through biomimetic approaches.27–31 Also, SF is able to control the growth and crystallization of calcium phosphate, as amorphous calcium phosphate (ACP) and hydroxyapatite (HAP) could be obtained by adjusting the mineralization time, the mass concentration of SF, and the molar concentrations of calcium ions and phosphate ions.32

In this work, we try to prepare a hybrid material with a controllable crystal morphology, and adjustable percentage of inorganic material for different demands. The SF based nanofibrils are assembled through a convenient and low-cost “ethanol-induced” method.33,34 These nanofibrils have excellent biocompatibility, tunable degradability and are expected to replace the collagen in bone repair materials. Then we prepared a SF nanofibril/nano hydroxyapatite (nanoHAP) film by vacuum filtration and an injectable hydrogel via a simple fibrillation and centrifugation approach, expecting to harvest the SF/HAP hybrids with a controlled polymorph of HAP, well-defined structures and various mechanical properties.

### Experimental

#### Preparation of regenerated silk fibroin (RSF) aqueous solution

The Bombyx mori silk fibroin solution was obtained after degumming, dissolving and dialyzing processes in accordance with a typical protocol described in the available literature and procedures,27,31,32 yielding a solution with a protein concentration of approximately 4 wt%, and stored at 4 °C for further use. Although such a regeneration process was applied, the term silk fibroin (SF) is still employed in this work to refer to the regenerated silk fibroin (RSF).

#### Preparation of SF nanofibrils

As detailed in the literature,31,32 the SF solution was diluted to 0.75 wt%, and the pH adjusted to 9.5 with 0.5 mol L⁻¹ NaOH aqueous solution. Then ethanol was added with a final concentration of 7 vol%. The mixture was incubated at room temperature for about 3 days for the growth of SF nanofibrils.

#### Production of SF nanofibril/nanoHAP films

The representative production route of nanofibril/nanoHAP film is as follows. 0.2 mol L⁻¹ CaCl₂ aqueous solution was mixed with SF nanofibrils for 30 min, and then 0.2 mol L⁻¹ Na₂HPO₄ aqueous solution was added before mixing for another 30 min, according to the mass ratio of HAP and SF nanofibrils (0.05, 0.10, 0.15, 0.20, 0.25, and 0.40). The obtained SF nanofibril/nanoHAP suspension was incubated at 37 °C for 24 h. Finally, the films were fabricated by vacuum-filtrating the solution through filtration membranes (pore size: 0.2 µm) for 12 h and dried at room temperature. It should be noticed in this work that the HAP was the only polymorph of calcium phosphate which was confirmed by SAED and XRD, as shown in Fig. 1 and S2.† Therefore, we assumed that HAP was quantitatively formed in the solution of SF nanofibrils due to it being nearly insoluble in alkaline conditions. This assumption has been briefly checked by TGA. Therefore, all the SF/HAP ratios were calculated according to the mass ratio of calcium/phosphate and SF nanofibrils.

#### Production of SF nanofibril/nanoHAP hydrogel

The representative production route of SF nanofibril/nanoHAP hydrogel is as follows. 0.2 mol L⁻¹ CaCl₂ aqueous solution was mixed with SF nanofibrils for 30 min. Then 0.2 mol L⁻¹ Na₂HPO₄ aqueous solution was added before mixing for another 30 min, according to the mass ratio of HAP and SF (0.05, 0.10, 0.15, 0.20, 0.25, and 0.40). The obtained SF nanofibril/nanoHAP suspension was incubated at 37 °C for 24 h. To remove the “free” Ca ions, the suspension was dialysed in de-ionized water for 12 h before it was further used. Afterwards, 0.6 mL aliquots of NaCl solution with different concentrations were separately added into 5.4 mL of SF nanofibril/nanoHAP
Characterization of SF nanofibril/nanoHAP hybrids

TEM images were obtained on a CM 20 (Philips) transmission electron microscope at an accelerating voltage of 100 kV. One drop of the nanofibril/nanoHAP mixture was cast on a copper grid with a carbon support film. The superfluous mixture was removed by a filter paper in 30 s.

Scanning electron microscopy (SEM) observation was performed with a TESCAN TS5136 MM at 20 kV of accelerating voltage. The nanofibril/nanoHAP mixture (dropped on a glass slide), the surface and cross-section of the films (Au-coated for 10 s) were imaged, respectively. A cross-section of the films was obtained by quenching in liquid nitrogen and then mechanical fracturing.

Powder X-ray diffraction (XRD) patterns were recorded on a Bruker D8 X-ray diffractometer (Germany) with Ni-filtered Cu Kα radiation (40 kV, 40 mA), applying a scanning rate of 0.02° s⁻¹ in the 2θ range from 10° to 80°.

All rheological tests were carried out at 37 ± 1 °C in strain controlled mode through a Physica MCR 301 rheometer (Anton Paar GmbH, Austria) operating in a 25 mm parallel-plate configuration with a 1 mm gap distance. 1 mL of hydrogel was dispensed on the bottom plate. To minimize water evaporation, low density mineral oil was added around the hydrogel on the plate. Frequency sweeps were performed over a frequency range from 0.1 to 10 Hz (strain 0.1%). As for the shear recovery test, the hydrogel was firstly stimulated at a 0.1% strain and increased to 5000% in 6 s, and finally returned to 0.1%. The cycle was repeated at least 7 times.

Biocompatibility of SF nanofibril/nanoHAP films

Biocompatibility evaluation of SF nanofibril/nanoHAP films was carried out by testing the cytotoxicity of the extraction medium using MC3T3-E1 cells according to the ISO standard (ISO10993.12-2004). The composite films were sterilized by soaking in ethanol aqueous solution (75 vol%) and then washed with PBS to remove the ethanol. Then the films were put into a culture plate with fresh culture medium. Dulbecco’s modified Eagle’s medium (DMEM, Gibco), supplemented with 10% fetal bovine serum, 100 U mL⁻¹ penicillin, and 100 μg mL⁻¹ streptomycin, was added at 1.25 cm² mL⁻¹ under sterilized conditions. The MC3T3-E1 cells were seeded into a 96-well tissue culture plate at a seeding density of 2000 cells per well in a CO₂ (5%) incubator at 37 °C. After incubation for 24 h, the pristine DMEM medium was replaced by the extracts of the composite films (200 μL per well). In the meantime, the DMEM medium in the control group was also replaced by fresh culture medium. After incubation for 24 h, 72 h, and 168 h, the cell metabolic viability was measured using CCK-8 assay on an Elx 800 instrument (Biotek, USA). The relative cell viability (%) was calculated as follows:

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\text{Cell viability} (\%) = \frac{[A]_{\text{test}}}{[A]_{\text{control}}} \times 100\% ,
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where [A]test is the absorbance of the test sample, and [A]control stands for the absorbance of the control sample.

Results and discussion

Composition and structure analysis of SF nanofibril/nanoHAP hybrids

The polymorphs and morphology of those calcium phosphates crystallized with the involvement of SF nanofibrils in the suspension were observed by TEM and SEM (Fig. 1). Compared with pure crystals in the control experiment (using a 0.15 wt% SF solution instead of SF nanofibrils, Fig. S1†), the inorganics precipitated within the organic matrix hold a deviated shape and have no ordered assembly behavior compared to Fig. 1. These differences clearly showed that the presence of SF nanofibrils influenced the crystallization and assembly of calcium phosphate. It also indicates interactions between silk nanofibrils and calcium phosphate particles, inducing in the organic/inorganic composite a “flower-like” morphology formed along the SF nanofibrils, with regard to the different inorganic contents. The “flower-like” crystals could be seen in samples with inorganic content (mass ratio of calcium phos-
phate and SF) below 0.15 (Fig. 1a and b). Whereas in the samples with inorganic content up to 0.2 and 0.25 (Fig. 1c–f), these “flower-like” crystals began to merge with each other, whose dimensions and shapes were much like those of the mineral phase present in natural bone.30

Additionally, when the ratio was raised to 0.4, there would be many more merged “flower-like” crystals as well as the appearance of the single pieces of crystal in the view (Fig. 1g and h). Indeed, the “flower-like” nanocrystals were well dispersed in the matrix. We presume that the emergence of these “flower-like” calcium phosphate crystals in the presence of SF nanofibrils might relate to the chelation between Ca2+ and acidic amino acid residues on SF nanofibrils. By increasing the ionic strength (e.g., CaCl2), the Debye length of the surface charge on SF nanofibrils becomes shorter, reducing the repulsive force between the SF nanofibrils, resulting in a slight aggregation among SF nanofibrils. At a low inorganic ratio (e.g., 0.05, 0.1, and 0.15), the crystals had ample space to grow, which led to “flower-like” morphology. While at higher ratios (e.g., 0.2 and 0.25), the space for growth was filled with more crystals that tended to merge with each other. Furthermore, the aggregates became the nucleation sites of HAP, forming organic/inorganic composite hybrids at the nanoscale. From the SAED pattern of the crystals, the mineral phase might be defined as HAP because its polycrystalline rings of the (211) and (002) planes could be observed (Fig. 1e insert). On the other hand, there were limited acidic groups on the SF nanofibrils. With the Ca2+ concentration increasing to a superfluous level (e.g., inorganic content of 0.4), the calcium and phosphorus could not chelate with nanofibrils, and those free ions would form larger pieces in the solution.

The polymorph of calcium phosphate mediated by SF nanofibrils was further detected by XRD. As shown in Fig. S2,† the (211) and (002) diffraction peaks of HAP centered at around 32° and 26°, respectively, were measured from the hybrid films. SF molecule chains behaved differently from SF nanofibrils when the crystals were modulated. Unlike silk fibroin molecules, which would produce amorphous calcium phosphate (ACP) in certain condition,32 only HAP was provided under the modulation of silk nanofibrils. These characteristic peaks gradually became more acute. Since HAP was the only inorganic component in the hybrid, more acute peaks indicated that the content of HAP rose with the increase in the mass ratio. The XRD spectra of the nanofibril/nanoHAP composites illustrated the presence of HAP as the unique crystalline phase (PDF Card No. 9-432).35,36 Whereas, overlap and extensive broadening of the diffraction peaks were detected, as in the case of natural bone, which suggested that the crystallinity of the HAP crystals was not very high.37

In the experiment, if we kept increasing the ratio of calcium and phosphorus sources, macroscopic pieces of HAP would be obtained (Fig. S3†), indicating that the SF nanofibrils could colloidaly disperse and stabilise HAP at both nano-scale and macroscopic-scale.

**Morphology and biocompatibility of SF nanofibril/nanoHAP hybrids**

Fig. 2 illustrates the route to generate the SF nanofibril/nanoHAP films. An SF solution was obtained after degumming, dissolving and dialyzing processes in accordance with a typical protocol described in the available literature.38,39 When the SF solution, which was adjusted to pH 9.5 and mixed with ethanol in advance, was incubated for one day at room temperature, the SF molecules underwent a proper conformation transition induced by ethanol (i.e., from random coil to β-sheet) into self-assembled elongated nanofibrils.31 Different amounts of CaCl2 aqueous solution were added to the SF nanofibril suspension under stirring to make sure the calcium ions had good binding with those nanofibrils due to the presence of electrostatic interactions (the isoelectric point of SF is 4.53, and SF nanofibrils were negatively charged at high pH value).40,41 Then Na2HPO4 aqueous solution, according to the targeted HAP contents, was mixed for another 30 min. After the obtained suspension was incubated at 37 °C for 24 h, the films were fabricated by vacuum-filtrating42,43 the suspension through filtration membranes. Indeed, when the mass ratio of nanoHAPs and SF nanofibrils was lower than 0.25, self-supporting films could be obtained. However, keeping increas-

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**Fig. 2** Schematic representation of the procedure followed to prepare silk fibroin nanofibril/nanoHAP films and the resultant photograph.
ing the content of nanoHAPs might damage the integrity of the film, because an overdose of the inorganic part (HAP) might influence the interactions among the organic part (SF nanofibrils) (Fig. S4†).

The SEM micrographs shown in Fig. 3 reveal the surface and cross-section morphology of the pure SF nanofibril and nanofibril/nanoHAP films. The pure SF nanofibril film displayed nearly full density and a smooth surface (Fig. 3a). However, the introduction of HAP evidently influenced the surface morphology of the films, as they presented coarse surfaces with a porous structure that became rougher with an increase in the inorganic component (Fig. 3c, e and S5b–f). It was speculated that the increased HAP content gradually weakened the intermolecular interactions within the SF nanofibril network, which led to alterations in the microstructures of the films. Similar morphologies could be imaged from the cross-section of nanofibril/nanoHAP films (Fig. 3d, f and S6†). Furthermore, it was found that almost nanoHAP particles were well-dispersed in the SF nanofibril substrate and no obvious silk–HAP interface was observed. Therefore, it was demonstrated that the combination of the in situ production of HAP mediated by SF nanofibrils and the vacuum filtration technique in this work efficiently achieved SF nanofibril/nanoHAP films with a uniform microstructure. Additionally, an elemental analysis of these nanofibril/nanoHAP composite films was performed to show that the molar ratio of calcium and phosphorus was 5:3, which also confirmed that the minerals produced were hydroxyapatite.8 Also, as shown in Fig. S7† the SF nanofibril/nanoHAP films have an elastic modulus of ca. 8.9 MPa, which is of the same magnitude of that of cancellous bone with values between 10 and 50 MPa. This indicated that the film might not only play a role as a porous material in damaged bones to some extent but might also have similar minerals to help in the bone repairing process.

It is important for biomedical materials to be stable and to maintain their integrity in bone repair. The porous structure of the SF nanofibril/nanoHAP films was likely to be favorable to the cells adhering and might have some merits in the circulation of body fluids and blood.

Fig. 3 Surface (a, c, e) and cross-section (b, d, f) morphology of SF nanofibril/nanoHAP films with different theoretical contents of hydroxyapatite. Mass ratio of HAP and SF: a and b, 0; c and d, 0.1; e and f, 0.2.

We kept the hybrid films soaking in 0.01 mol L⁻¹ PBS solution (pH = 7.2), 37 °C for 14 days, and it turned out from Fig. S8 and S9† that the integrity and weight barely change. At microscopic scales, XRD patterns of SF nanofibril/nanoHAP hybrid films immersed in PBS for 14 days (Fig. S8a†) or even longer were similar to those of as-prepared hybrids in Fig. S2† (the position of the peaks and the peak width at half height did not change), indicating that the obtained HAP were stable in aqueous solution.

Furthermore, a CCK8 assay was carried out to evaluate the cytotoxicity of SF nanofibril/nanoHAP hybrid films on MC3T3-E1 cells. As shown in Fig. 4, the cell viability were all over 96% after culturing for 1, 3, and 7 days, indicating that the biocompatibility of hybrid films meets the common requirements.18

Viscoelastic behaviour and stability of injectable SF nanofibril/nanohydroxyapatite hydrogels

On the other hand, nanofibril/nanoHAP hybrids can be made into thixotropic hydrogels via a simple one-step centrifugation procedure, which has previously been employed to produce a pure SF nanofibril hydrogel with thixotropy.40 Furthermore, the mechanical (rheological) properties of such SF nanofibril-based hydrogels could be easily controlled by adjusting the contents of salt (NaCl in our case).

Electrostatic interactions among SF nanofibrils would be suppressed when increasing ionic concentrations were applied to
the nanofibril/nanoHAP hybrids, correspondingly increasing the solid content of the hydrogel, as illustrated in Table S1.†

In order to investigate the mechanical strength (viscoelasticity) of the SF nanofibril/nanoHAP composite hydrogels, rheological tests were performed, as shown in Fig. 5. With the increment in the concentration of NaCl from 25 to 125 mmol L\(^{-1}\), the storage modulus (\(G'\)) of SF nanofibril/nanoHAP hydrogels increased from 4500 to 10 000 Pa, which meets the mechanical requirement for biomedical injectable hydrogels.42 In addition, the proportion of inorganic and mechanical properties of the hydrogel could be adjusted as needed for various demands. As for a thixotropic hydrogel, its injectable properties should be assessed by some crucial critical parameters like the recovery rate and ratio. Outstandingly, after a large strain treatment (5000%), the storage modulus (\(G'\)) of SF nanofibril/nanoHAP composite hydrogel could recover to 85% in 50 seconds, and maintain the rate and the ratio after continuous repeats (7 times).

Finally, the stability of the hydrogel was verified. The sample was immersed in 0.01 mmol L\(^{-1}\) PBS (pH = 7.2, 37 °C, NaCl 150 mmol L\(^{-1}\)). As shown in Table S2 and Fig. S10,† the solid content and the mechanical properties of the hydrogel were retained.

Therefore, the thixotropic property, the fast and good recovery, as well as the ideal stability of the hydrogel, suggest the material might have promise in bone tissue repair, especially after irregular damage. After being injected, the hydrogel was expected to remain in the damaged area and remain as a calcium source during convalescence.

As for the possible biomineralization and gelation mechanism: first, carboxyl on the SF molecules could chelate Ca\(^{2+}\), which became a nucleation site and made HAP grow on the SF nanofibrils in situ. Then when NaCl was added, the Debye length (attributed to the existence of electrostatic interactions) between SF nanofibrils was reduced, resulting from the SF nanofibrils/nanoHAP hybrids becoming easily entangled to form three-dimensional networks under centrifugation. In contrast, an SF nanofibril solution without the presence of NaCl is stable under centrifugation or other shearing actions. More importantly, the first step would not bring in other impurities, except hydroxyapatites which grow in situ and sodium chloride, which helped to stabilize the hydrogel.

Unlike their injectable counterparts, these SF nanofibril-based hydrogels displayed better thixotropic properties, a more uniform nanofibrillar network, lower solid content and good biocompatibility, especially because they contain nanoHAP as a resource of Ca\(^{2+}\) and PO\(^{4-}\). Therefore, they are expected to have advantages in repairing irregular damage in bones.

**Conclusions**

In this study, materials with potential for bone regeneration were fabricated through an “in situ” biomineralization process. Considering the similarities of SF nanofibrils and collagen fibrils, which are the main organic component in the structure of natural bone, mechanical performance and biocompatibility, as well as the homogeneously nanofibrillar network which was important for encapsulated cells, we employed SF nanofibrils to mediate the mineralization of HAP with a controllable morphology and size. It was found that the “flower-like” nanoHAP could be formed in situ on silk nanofibrils via the strong interaction between two biocompatible components. The morphological observation illustrated that nanoHAP particles were well dispersed and stabilized in the organic sub-strate. “Hard” SF nanofibril/nanoHAP hybrid films with various HAP contents were prepared by the in situ production of HAP on SF nanofibrils and vacuum filtration. When HAP was involved, the films turned microporous and became rougher in relation to the amount of inorganic component. Furthermore, cytotoxicity results proved the biocompatibility of such hybrids. At the same time, a “soft” nanofibril/nanoHAP hydrogel could be prepared via a simple centrifugation method with the assistance of NaCl. Hydrogels with an ECM-like structure represented not only adequate viscoelastic properties, but also remarkable thixotropy, with a storage modulus (\(G'\)) able to

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**Fig. 5** The rheological properties of injectable SF nanofibril/nanoHAP hydrogel. (a) Storage modulus (\(G'\)) and loss modulus (\(G''\)) versus frequency for the hydrogel with NaCl concentration of 25, 50, 75, 100, 125 mmol L\(^{-1}\) at 37 °C. (b) Shear recovery test of SF the hydrogel with 125 mmol L\(^{-1}\) NaCl at 37 °C. It took around 50 s to recover the hydrogel behavior after shearing with 5000% strain for 6 s.
recover to 85% within 50 seconds when a great shearing strain (5000%) was applied. Meanwhile, nanoHAPs grew in situ in the hydrogel, which united the excellence of properties, structure and processing, and were expected to show promise in implanted biomaterials and bone tissue repair.

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Notes and references