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Thixotropic silk nanofibril-based hydrogel with extracellular matrix-like structure

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ABSTRACT:

Silk fibroin (SF) based materials are widely studied and applied in bio-related fields due to their excellent structural and biological properties. Here, we present an injectable hydrogel formed by SF nanofibrils via simple fibrillation and centrifugation approach. The hydrogels with extracellular matrix-like structure not only perform the sufficient mechanical properties, but also show outstanding thixotropic character, whose storage modulus (G') can recover to 93% within 40 seconds after a striking injection. More importantly, the injectable hydrogel exhibits significant biocompatibility for L929 cells cultured in hydrogel after injection, illustrated by cell viability and cytotoxicity assays. All of these results indicate that such SF nanofibril-based hydrogel has promise in application as cell therapy carrier.

Injectable hydrogels are extensively studied as ideal implanted biomaterials for cell therapies in tissue engineering and regenerative medicine,[1,2] since a) injectable matrix can appropriately fill the irregularly shaped tissue defects with minimal surgical wounds and patient discomfort;[3] b) drugs or cells can be incorporated into the hydrogel simply by mixing before injection;[4,5] c) these water-holding three-dimension networks resembling extracellular cell matrix (ECM) possess high permeability for oxygen, nutrients, waste metabolites and intercellular chemical signaling.[6,7] However, a comprehensive and systematic approach to gain such hydrogel with balanced processing-structure-function relationships remains challenging.[8-10]

From the aspects of processing, an environmentally friendly and facile approach for obtaining low-cost injectable hydrogel is still to be pursued in both chemical and physical gelation fields.[11,12] Compared with the chemical gelation that normally requires elaborate methods, harsh conditions and cytotoxic solvents in the processing, physical gelation is more reliable to address these issues due to the moderate and cytocompatible preparation conditions such as temperature and pH. In the meantime, the strategy to construct biomacromolecularly nanofibrillar hydrogels with ECM-like structure is still in its infancy. For example, only cellulose nanofibril-based hydrogel started to emerge recently.[13] The critical roadblock is that ECM is universally hierarchical at a multitude of diverse scales like other biological tissues and materials.[14-16]

According to the flowing state before injection, injectable hydrogels may be divided into two categories. The common one is that it is a solution before injection

and becomes gel at the defected tissue *in situ*.[4] Another is a preformed solid-hydrogel holding shearing-thinning and self-healing characters (so called thixotropy), and can be delivered by syringe.[17-19] Obviously, the latter may have better way to prevent unwanted leakage due to insufficient gelation rate.[20]

Silk fibroin (SF) is an ideal candidate for achieving the injectable hydrogels, not only because of its excellent biocompatibility and tunable degradability[21-23], but also due to its strong self-assembly tendency as well as facile processibility.[24-26] Zhang *et al.* reported that they can obtain the aqueous dispersion of SF nanofibrils directly from natural silkworm cocoon silks.[27] However, the well-dispersed SF nanofibril solution made from SF aqueous solution has seldom been reported.[28,29] That probably results from the high instability of SF solutions, which show a strong tendency to form hydrogels or aggregates at environmental conditions with low pH,[30,31] high temperature,[32] shear forces,[33] and alcohol treatment.[34] Recently, we found that SF molecules could assemble into elongated nanofibrils comparable to amyloid nanofibrils.[29] In this study, we show that such a SF nanofibril solution can be further converted to an injectable hydrogel with thixotropic property through a simple one-step centrifugation process. The mechanical strength of such a SF nanofibril-based hydrogel can be tuned handily by adjusting the sodium chloride content added in the system. Compared with previous silk injectable hydrogels, SF nanofibril-based hydrogels show higher thixotropy, lower solid content and more homogeneously nanofibrillar network (ECM-like structure), so such hydrogels can be applied for cell carrier with remarkable cell encapsulation and delivery ability.

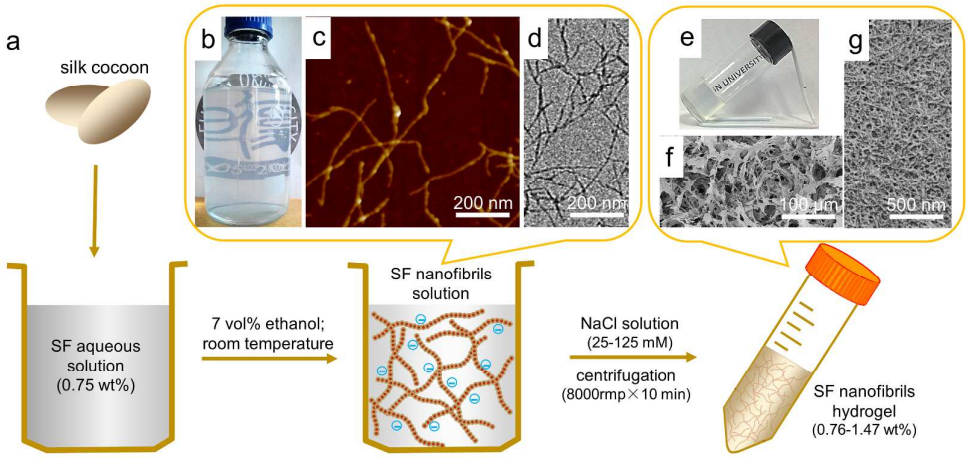

Fig. 1 Fabrication of SF nanofibril-based hydrogels with ECM-like structure. (a) Schematic representation of the procedure followed to prepare SF nanofibril-based hydrogels. SF solution (0.75 wt%) was prepared from *B. mori* silkworm cocoon, then incubated such solution to form SF nanofibrils in 7 vol% ethanol solution at room temperature 1 day. Finally, SF nanofibril-based hydrogel was prepared simply via centrifugation with NaCl in solution. **(b)** The resultant SF nanofibril solution with opalescence. **(c,d)** AFM and TEM images of SF nanofibrils. **(e)** SF nanofibril-based hydrogel. **(f,g)** SEM images of lyophilized SF nanofibril-based hydrogel with different magnifications, which show ECM-like structure.

The approach followed to generate the SF nanofibril-based hydrogel is sketched in Fig. 1a. First, *Bombyx mori* silkworm cocoon silk fibers were degummed, dissolved and dialyzed in accordance with a typical protocol described in available literature and supporting information,[26] yielding a solution of protein concentration of ca 5 wt%. The solution was further adjusted to 0.75 wt% and pH 9.5 with 0.5 mol/L NaOH aqueous solution and then mixed with ethanol (the final concentration of ethanol 7 vol%). By incubating this solution at room temperature over 1 day, the SF molecules self-assembled into elongated nanofibrils due to appropriate conformation transition of SF induced by ethanol. The obtained SF nanofibril solution (0.14 wt%) is opalescent (Fig. 1b), and those SF nanofibrils are up to 1 μm long regarding to their contour length observed by AFM and TEM (Fig. 1c and 1d), which are identical with

our previous findings.[29] CD curve of SF nanofibril solution, as well as the FTIR spectrum (sharp absorption at 1623 cm^{-1}) of these dried SF nanofibrils prove that β-sheet is the dominant conformation in such nanofibrils (Fig. S1†).[35] On account of the existence of electrostatic interactions (the isoelectric point of SF is 4.53,[36] and SF nanofibrils were negatively charged at basic pH, which is confirmed by the Zeta-potential test. For example, the value is -18 mV at pH=6.8.) and bactericidal effect (ethanol) in solution, this SF nanofibril solution can stabilize for more than 6 months without precipitation and mildew, as shown in Fig. 1b.

ECM is an intricate network composed of nanofibrillar proteins (mostly collagen).[16] Compared with the diameter and length of constituent nanofibrils, the resultant nanofibrillarly 3D network is more important for encapsulated cells.[13,16] Considering the structural, mechanical and biocompatible similarities between SF nanofibrils and collagen fibirls,[37,38] as well as inherent shear thinning properties of nanofibrillar network,[39] we set out to prepare ECM-like injectable hydrogel via simple one-step centrifugation process. To prevent the negative effect of ethanol on cells, we have removed ethanol by dialyzing SF nanofibril solution against deionized water for 3 days before fabricating hydrogels. Initially, we found that electrolyte could induce SF nanofibrils to aggregate, as shown in Fig. S2†. Therefore SF nanofibril solutions (0.14 wt%) with or without sodium chloride are attempted to produce hydrogels. In the case containing NaCl, 0.6 mL NaCl solution with calculated concentration was mixed with 5.4 mL SF nanofibril solutions to give final solutions of 25, 50, 75, 100, 125 mmol/L NaCl separately, and these ion concentrations have no significantly

adverse effects on cells growth and proliferation.[20] No hydrogel could form from the SF nanofibril solution without NaCl, nevertheless SF nanofibril solution with NaCl (for all cases) formed a highly transparent hydrogel after only 10 min centrifugation with 8000 r/min, as shown in Fig. 1e, whose transmittance is up to 72% at 700 nm (Fig. S3†), close to that of cornea.[40] The corresponding SEM images (Fig. 1f and 1g) reveal that the resulting hydrogel is closely similar to ECM, displaying uniform porous and fibrous scaffold structure, which could offer a structural support for cells and potentially enhance cell adhesion, proliferation and absorb proteins and growth factors.[41,42]

As for the difference caused by NaCl in forming hydrogels, it could be a consequence of the Debye length variation. Due to the presence of electrostatic interaction, SF nanofibril solution is stable under the centrifugation or other shearing actions (same as the reason why SF nanofibril solution can stabilize for more than 6 months at room temperature, as mentioned before). In contrast, by adding NaCl, the Debye length of the surface charge on SF nanofibrils becomes shorter, therefore leading to reduced repulsive force between the SF nanofibrils (Fig. S2†). Finally, SF nanofibrils are easily entangled to form three dimensional networks under centrifugation.

According to our hypothesis, the repulsive force among SF nanofibrils (which can be easily manipulated by applying different NaCl concentration before gelation) has a critical influence on solid content of the hydrogels (Table 1), and thus will be directly reflected in their mechanical strength. Rheological experiments were

performed to evaluate the mechanical properties (viscoelastic behavior) of the SF nanofibril-based hydrogels, as shown in Fig. 2. The relative strain sweep and shear sweep curves are shown in Fig. S4†. Indeed, by progressively increasing NaCl from 25 to 125 mmol/L, the storage modulus (G') of SF nanofibril-based hydrogels can be increased substantially from 1100 to 3700 Pa. As we know, 480 Pa already meets the mechanical requirement for an implanted hydrogel,[43] thus the hydrogels in our case have tunable mechanical properties for different clinical requirements. In view of the physiological salt concentration (150 mM), we studied the variation of solid content and G' after SF nanofibril-based hydrogels were soaked in such NaCl concentration PBS. Fig. S5† illustrates that solid content and G' of all hydrogels increased, which could further prevent the possible leakage of hydrogel at defected tissue.

Table 1 The solid content of SF nanofibril-based hydrogels.

NaCl concentration (mmol/L)	25	50	75	100	125
Solid content (wt%)	0.76±0.07	1.01±0.05	1.19±0.01	1.32±0.07	1.47±0.15

Recovering rate and ratio are crucial parameters to evaluate the injectable properties of a thixotropic hydrogel as well. Remarkably, SF nanofibril-based hydrogel withstanding a great strain treatment (5000%), G' could recover to 90% only within 40 seconds, even after 7 times continuous repeats. To our knowledge, no such ultrahigh recoverability of pristine SF hydrogel has been reported previously. It also means that SF nanofibril-based hydrogel can effectively prevent the possible leak due to the inadequate gelation rate. This was confirmed by our tentative test. The hydrogel can be easily injected into desired styles, as shown in Fig. 3a, and meanwhile, the

shape of injected hydrogel can be fixed perfectly (see SI video).

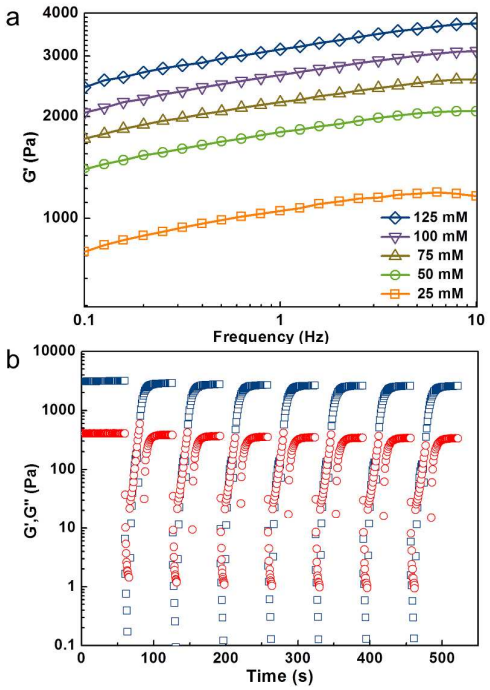

Fig. 2 The mechanical properties of injectable SF nanofibril-based hydrogels. (a) Storage modulus (G') versus frequency for SF nanofibril-based hydrogel samples with NaCl concentration of 25, 50, 75, 100, 125 mmol/L at 37 ºC. **(b)** Shear recovery test of SF nanofibril-based hydrogel with 125 mmol/L NaCl at 37 ºC.

Besides the structure and mechanical properties, biocompatibility is the most important respect for injectable hydrogels. Here, L929 cells were employed to evaluate the viability and cytotoxicity of the SF nanofibril-based hydrogels via CCK-8 assay. Fig. 3b presents the results of cytotoxicity test for SF nanofibril-based hydrogels at 1 and 3 days of incubation. It can be found that all samples' relative cell growth is more than 94%, clearly proving that there is no cytotoxicity for all SF nanofibril-based hydrogel samples. In order to demonstrate the biocompatibility and explore the potential application in cell-carrier field, L929 cells were encapsulated in the SF nanofibril-based hydrogel for a scheduled time (1, 3 and 7 days in our cases), and a corresponding Live/Dead staining was followed. It should be mentioned at this

stage that SF nanofibril-based hydrogels are no collapse in all of incubation times, and there is no detectable degradation in 7 days (Fig. S6†). The confocal images (Fig. 3d, 3f and Fig. S7†) reveal that cells showed uniform dispersion in the SF nanofibril-based hydrogels and demonstrate a similar cytotoxicity result to that of CCK-8 assay shown in Fig. 3b. More commendably, L929 cells showed spreading trend in the hydrogel (Fig. 3c and 3e), which may be due to the low hydrogel solid content and homogeneously nanofibrillar network. It also means that cells behave analogously with their native state in ECM. Given the excellent injectable property, we are confident that these SF nanofibril-based hydrogels will offer benefits for cell therapy.

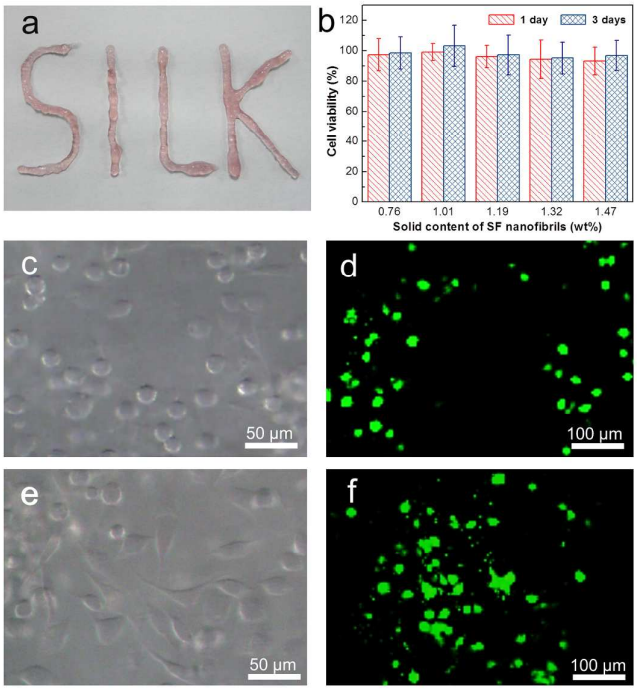

Fig. 3 The biocompatibility of injectable SF nanofirbril-based hydrogels. (a) SF nanofibril-based hydrogel loading cells after injection, which can be injected to preconceived style. The hydrogel was mixed with cell suspension. **(b)** The cytotoxicity of SF nanofibril-based hydrogels for L929 cells via CCK-8 assay. **(c-f)** The microphotographs **(c,e)** and fluorescent images **(d,f)** of L929 cells encapsulated in SF nanofibril-based hydrogel after 1 **(c,d)** and 3 days **(e,f)** of culturing time.

In summary, we prepared a new injectable SF nanofibril-based hydrogel via a facile centrifugation approach. Such a hydrogel shows similarly hierarchical structure with ECM. More importantly, this material has super resistance to ultrahigh shear, and meanwhile can maintain the outstanding recovering ability under iteratively ultrahigh shears. Finally, the cytotoxicity and biocompatibility results prove that SF nanofibril-based hydrogel is a superb hydrogel for implanted biomaterials. These injectable SF nanofibril-based hydrogels, which combined the advantages of processing, structure, and properties, have promise to be used in cell-carrier for different kinds of clinical therapy.

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Table of Contents