

Honeysuckle flowers extract loaded *Bombyx mori* silk fibroin films for inducing apoptosis of HeLa cells

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Abstract

This study aimed to prepare silk fibroin (SF) films loaded with honeysuckle flowers extract (HFE) for inducing apoptosis of HeLa cells. We mixed solution of SF and HFE by air-drying for preparing the honeysuckle flowers extract loaded silk fibroin (SFH) films. The physical properties including morphologies, contact angle, roughness, and Z range were characterized. MTS assay and fluorescence micrographs proved that SFH films inhibited the proliferation rate of HeLa cells due to induction of HFE into SF films. Furthermore, cell apoptosis assay and cell cycle analysis confirmed that the apoptosis of HeLa cells resulted from SFH films. Therefore, SFH films designed in our study might be a promising candidate material for cancer therapy.

KEYWORDS

biomaterials, cancer, Chinese herbal medicine

1 | INTRODUCTION

Silk fibroin (SF) derived from the *Bombyx mori* (*B. mori*) cocoons has excellent properties, including controllable biodegradability, biocompatibility and environmental stability (Gil, Panilaitis, Bellas, & Kaplan, 2013; Xu, Shi, Yang, Zhang, & Zhu, 2015; Yang, He, Shuai, Min, & Zhu, 2013). SF can be used to prepare various material formats including films (Coburn, Na, & Kaplan, 2015; Seib et al., 2015), hydrogels (Guziewicz, Best, Perez-Ramirez, & Kaplan, 2011; Kundu, Poole-Warren, Martens, & Kundu, 2012; Zhong et al., 2015), fibers (Dinis et al., 2014; Liu, Wang, Yao, Shao, & Chen, 2016) and microspheres (Srihanam, Srisuwan, Imsombut, & Baimark, 2011; Wen et al., 2011) under mild

processing conditions. This allows SF to be utilized as a promising material for biomedical applications such as drug delivery (Coburn et al., 2015; Wongpinyochit, Uhlmann, Urquhart, & Seib, 2015; Wu et al., 2016), tissue engineering (Han et al., 2015; Liu, Bai, & Zhao, 2014), and wound repair (Jin et al., 2014; Xu et al., 2015).

On the other hand, honeysuckle has been applied for long history as a commonly used veterinary medicine. Honeysuckle flowers (HF) are the dry flower buds or early opening flowers of *Lonicera japonica* Thunb in the *Caprifoliaceae* family (Lei et al., 2016). These flowers are abundant in organic acids, flavonoids, volatile oil, terpenoids, and inorganic elements. They showed pharmacological effects, such as antibacterial and antiviral properties, heat-clearing, and

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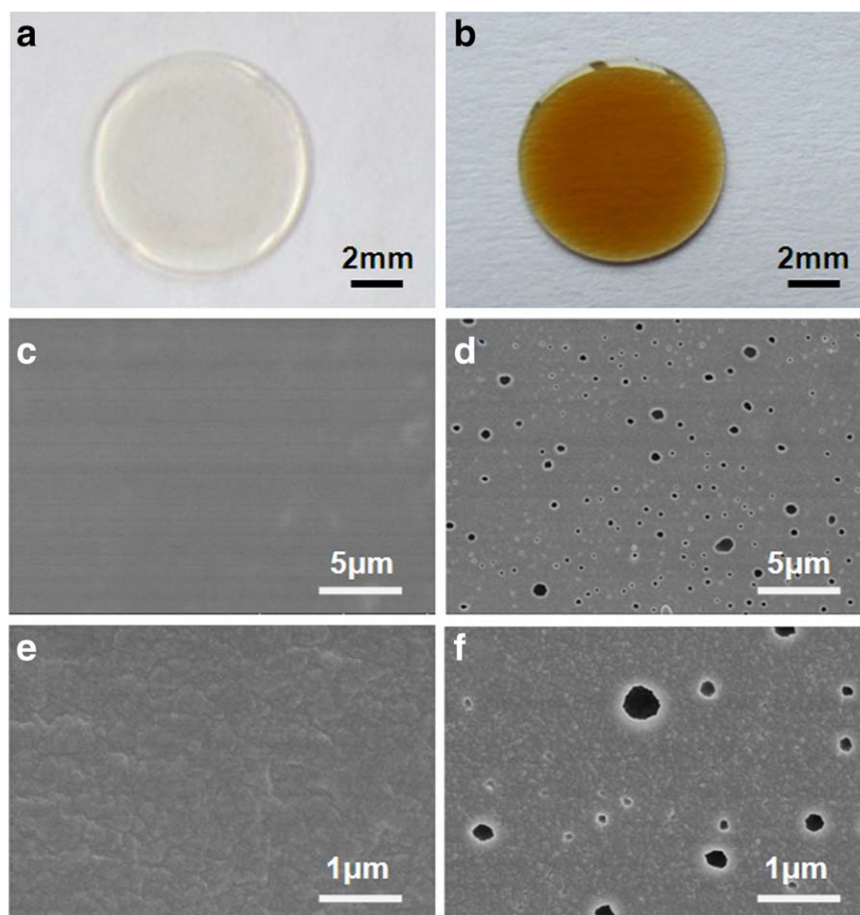


FIGURE 1 The SF film and SFH film (a, b). SEM micrographs of SF films (c, e) and SFH films (d, f). [Color figure can be viewed at wileyonlinelibrary.com]

detoxifying, protection of the liver and gallbladder, resistance to inflammation, lowering of blood lipid levels, and blood pressure (Gan et al., 2014; Lei et al., 2016). Increasing evidence has demonstrated that compositions and microstructures of materials used to support cancer cell growth play an important role in the fate of cancer cells (Gopal, Sita, Barbara, Valery, & Mao, 2010; Qiu et al., 2013). Previous research suggests that the mainly active ingredients of honeysuckle are chlorogenic acid and luteolin (Zhang et al., 2014). It is reported that chlorogenic acid killed pathogenic bacteria strains (*Shigella dysenteriae* and *S. pneumoniae*) by promoting irreversible permeability changes in cell membrane. This led cells to lose the ability to maintain membrane potential and released cytoplasm macromolecules including nucleotide (Lou, Wang, Zhu, Ma, & Wang, 2011). Therefore, HFs is a potential candidate to be made for anti-cancer drugs.

To our best knowledge, there is no report about anticancer drug delivery of using SF as a drug carrier for honeysuckle flowers extract (HFE) delivery. For achieving this goal, we attempted to mix SF aqueous solution and HFE to prepare HFE loaded SF films. A 20-min steam treatment was used to make film stable. Then films were cultured with HeLa (Henrietta Lacks) cells, AO-EB double staining method and MTS assay were performed on cells to verify cell viability. Apoptosis assay and cell cycle analysis was used to analyze cell apoptosis.

2 | MATERIALS AND METHODS

B. mori silkworm cocoons were purchased from Zhejiang Academy of Agricultural Sciences (China). HFE were provided by a friend in Tongde Hospital of Zhejiang Province (China). HeLa cells were purchased from Shanghai Institute of Life Sciences Research, Chinese Academy of Sciences. Na_2CO_3 and LiBr of analytical grade were purchased from Aladdin Chemical Reagents, China. Deionized water was used throughout the experiment. Dulbecco's modified Eagle's medium (DMEM) and 1% penicillin-streptomycin were purchased from Gibco.

2.1 | Preparation of aqueous *B. mori* SF solution

The aqueous SF solution was prepared according to procedure described previously (Rockwood et al., 2011). Briefly, *B. mori* cocoons were cut into small pieces and degummed in Na_2CO_3 aqueous solution (0.5%) at 100°C for 30 min and then rinsed thoroughly with deionized water to remove glue-like sericin protein. This degumming process was repeated twice. After washing and drying, the silk fibers were then dissolved in 9.3M LiBr solution at room temperature, followed by dialysis with cellulose tubular membranes (molecular weight cutoff, 3500 Da) against deionized water for 3 days to remove the LiBr salt. The

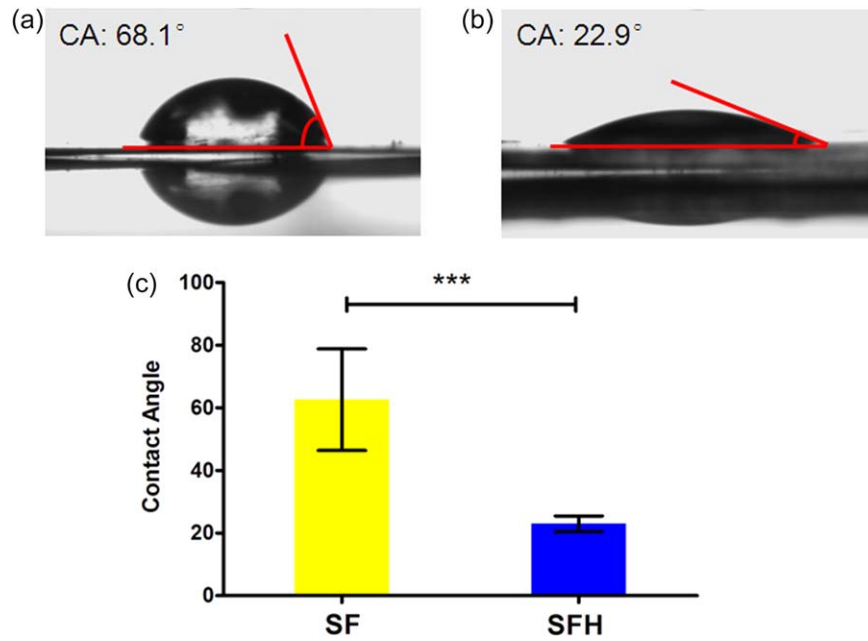


FIGURE 2 Water contact angle (CA) images of SF films and SFH films (a, b); histograms of CA (c). One-way ANOVA followed by Bonferroni's *post hoc* test, *** $p < .001$, \pm SD, $n = 3$ [Color figure can be viewed at wileyonlinelibrary.com]

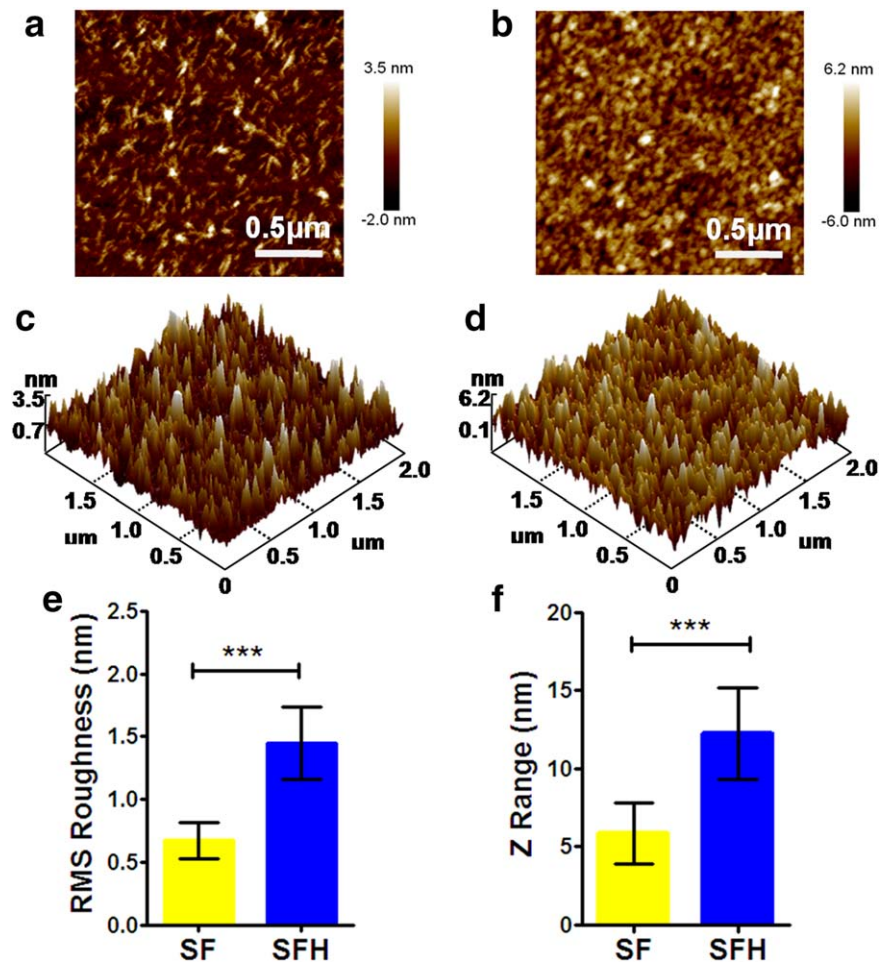


FIGURE 3 AFM images and corresponding section analysis of SF films and SFH films: (a) SF, (b) SFH, (c, d) three-dimensional images corresponding to (a, b) respectively; (e, f) histograms of RMS roughness and z range. One-way ANOVA followed by Bonferroni's *post hoc* test, *** $p < .001$, \pm SD, $n = 8$ [Color figure can be viewed at wileyonlinelibrary.com]

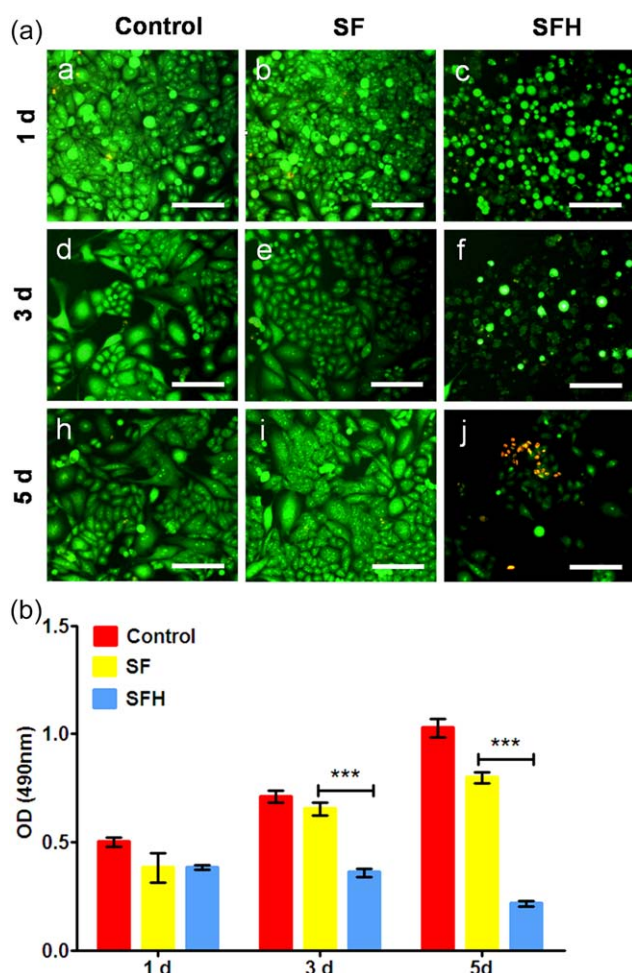


FIGURE 4 (a) Morphology of HeLa cells with SF films and SFH films, with nuclei of normal cells stained in green by AO and nuclei of apoptotic or dead cells stained in red by EB after being cultured for 1, 3, and 5 days. Scale bar: 200 μm. (b) MTS assay of HeLa cells proliferation with SF and SFH films after being cultured for 1, 3, and 5 days. One-way ANOVA followed by Bonferroni's *post hoc* test, *** $p < .001$, \pm SD, $n = 3$ [Color figure can be viewed at wileyonlinelibrary.com]

resulting aqueous SF solution was cleared by centrifugation and the final concentration of SF solution was calculated by weighing the remaining solid after drying. The concentration of aqueous SF solutions was 10 mg/mL for the following experiments.

2.2 | Preparation of HFE loaded SF films

The concentration of HFE was 1 g/mL. 50 μL SF solution was dropped on the roundish glass slide (diameter 0.8 cm) first, 20 μL honeysuckle flowers extract solution was added to the surface of roundish glass slide with SF solution on it. The film was air-dried at room temperature. A 20-min steam treatment was performed to induce structural transition of SF from random coil to Beta-sheet,¹ resulting in stabilized SF films loaded with HFE. The films loaded with HFE solution are denoted as SFH in following experiments.

2.3 | Characterization

Stabilized films were assembled on a metallic base, coated ingold, analyzed and photographed by scanning electron microscope (SEM, SU8010; Hitachi, Japan). And films were also observed with atomic force microscopy (AFM, MultiMode, VEECO, USA.) in tapping mode. The static water contact angle measurements were carried out both on SF films and SFH films by using a goniometer (OCA20, DataPhysics, Germany) with deionized water. For AFM, SF solution and the HFE solution were mixed at a ratio of 5:2, 200 times diluted by deionized water, and the final concentration of SF solution was 0.5 mg/mL, the final concentration of HFE solution was 5 mg/mL. 10 μL of this mixed diluted solution was deposited on freshly cleaved mica and air-dried, followed by a 20-min steam autoclaving cycle. The images were taken and processed by software (NanoScope Image).

2.4 | Cell morphology and proliferation

We used HeLa cells as testing cells. HeLa cells were cultured in DMEM with 10% FBS and 1.0% penicillin–streptomycin. Medium was changed every 2 days. Cells were cultured with SF films, SFH films for 1, 3, and 5 days, respectively. Cells cultured without films were used as control. HeLa cells (6500 cells/cm²) were seeded on 48-well microplates. After the cells were cultured for 1, 3, and 5 days, the cell morphology observation was performed by dyeing with acridine orange (AO) and ethidium bromide (EB) at a ratio of 1:1 for 5 min. Nuclei of normal cells were visualized by AO (Solarbio, China), and nuclei of apoptotic or dead cells were visualized by EB (Solarbio, China). The cells were viewed under Fluorescence Inversion Microscope System (Olympus IX51, Japan). In addition, Cell Titer 96 Aqueous OneSolution cell-proliferation (MTS) assay was conducted to verify the cell viability according to the manufacturer's protocol (Promega).

2.5 | Apoptosis assay

HeLa cells were cultured in 6-well plates at a density of 5.0×10^5 cells/well in triplicates and treated for 1, 3, and 5 days, respectively, with SFH films. Each SFH film consists of 400 μL SF solution and 160 μL HFE solution with a diameter of 2 cm. Two SFH films were put in each well. After cultured for 1, 3, and 5 days, cells were harvested and tested by apoptosis assay conducted by AnnexinV-FITC/PI Apoptosis Detection Kit according to the manufacturer's instructions (keyGEN BioTECH). Stained cells were analyzed with a flow cytometer (BD FACSCalibur, USA). As a protein with a molecular weight of 36.0 kDa, Annexin V has a high affinity for phosphatidylserine (PS) on the cell membrane. PS is exposed on the cell membrane when cells in early apoptosis (Honda, Zhao, & Kondo, 2002). FITC-/PI- denotes normal (nonapoptotic), FITC+/PI- denotes early apoptosis, and FITC+/PI+ stained cells are in secondary necrosis/late apoptosis (Koopman et al., 1994; Shi et al., 2016; Vermes, Haanen, Steffens-nakken, & Reutelingsperger, 1995).

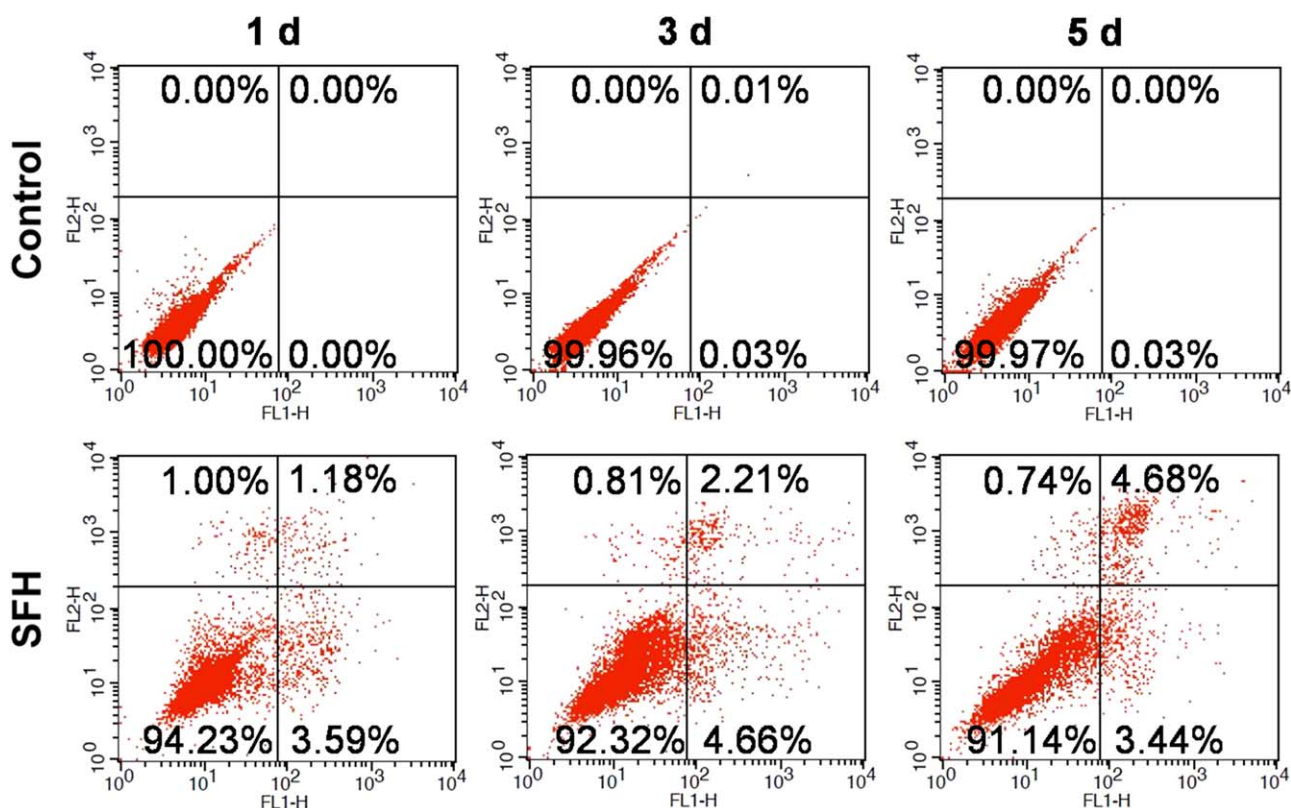


FIGURE 5 Effects of SFH films on introduction of HeLa cell apoptosis. The cells were cultured with SFH films for 1, 3, and 5 days, and collected and stained with FITC-conjugated Annexin V and PI. The percentage of apoptotic cells was calculated from the ratio of the sum of early and late apoptotic cells using the FCS-Express software. Each red dot represents for an Annexin V/PI stained cell [Color figure can be viewed at wileyonlinelibrary.com]

2.6 | Cellcycle analysis

The distribution of cells at different stages in the cellcycle was determined by flow cytometric (Asami, Takahashi, & Shirahige, 2000). Briefly, HeLa cells were cultured in 6-well plates at adensity of 6×10^5 cells/well in triplicates and treated for 48 h with SFH films. All the cells (both the adhered and floating cells) were trypsinized, harvested, washed twice with PBS, fixed in 70% ethanol at 10^6 cells/mL and stored in 4°C for at least 24 h. Then ethanol-suspended cells were diluted with PBS then centrifuged at 1000g for 5 min to remove residual ethanol. For cell-cycle analysis, the cell samples were suspended in 100 μ L RNase A (key-GEN BioTECH, Nanjing, China) for 30 min at room temperature and 400 μ L PI for 30 min in the dark at 4°C. A minimum of 10^4 cells per sample was evaluated, and the percentage of cells in each cellcycle phase was calculated using a flow cytometer (BD FACSCalibur, USA).

2.7 | Statistical analysis

Data were analyzed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, USA). Sample pairs were analyzed with the Student's t-test. Multiple samples were evaluated by one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test to evaluate the statistical differences ($p \leq .05$) among all samples. An asterisk denotes statistical significance as follows: * $p < .05$, ** $p < .01$, *** $p < .001$. All data presented are as mean values \pm standard deviation (SD).

3 | RESULTS AND DISCUSSION

3.1 | Characterization of SFH film

Morphological features of SFH and SF film were observed by SEM. As shown in Figure 1, holes of different size distributed evenly on the surface of SFH film while SF films rather flat and intact. And for static water contact angle (CA) measurements, CA of SFH films are much smaller than SF films' (Figure 2). It indicated that the addition of HFE greatly increased the hydrophilicity of films. In addition, AFM was performed to observe the nanotopography of SFH and SF films. Figure 3a and b showed that SF films with nanorod-like morphology turned to nanorods clusters after addition of HFE. Their corresponding 3D images of AFM indicated that SF and HFE were distributed homogeneously on films (Figure 3c,d). The statistical analysis of RMS roughness and Z range of the SF film and SFH film are indicated in Figure 3e and f, respectively. The RMS roughness of SF and SFH films was 0.67 ± 0.14 nm and 1.45 ± 0.29 nm, respectively. The Z range of SF and SFH films was 5.87 ± 1.94 nm, 12.24 ± 2.92 nm, respectively. This suggests that SFH film has rougher surface than SF film does due to induction of HFE in SF.

3.2 | Cell morphology and proliferation

To determine the effect of the SFH film on inhibiting cell growth, we seeded HeLa cells cultured with SF films and SFH films for observing

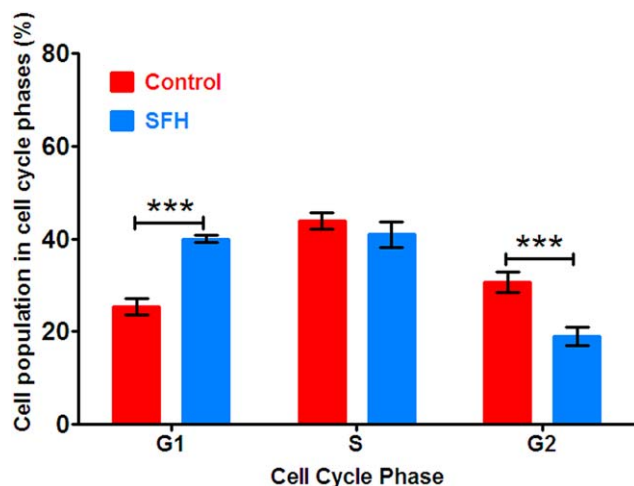


FIGURE 6 Effects of SFH films on cycle phase distribution of HeLa cells. Percentage of cell populations in G1, S, and G2 phases. One-way ANOVA followed by Bonferroni's *post hoc* test, *** $p < .001$, \pm SD, $n = 3$ [Color figure can be viewed at wileyonlinelibrary.com]

the morphology with immunofluorescence and testing cell proliferation by MTS assay. The fluorescence micrographs showed that cells cultured with SFH films occurred apoptosis since day 1 as cells' shape became round (Figure 4a). After cultured for 5 days, the amount of HeLa cells cultured with SF films is similar to that of control group, however, cells cultured with SFH films were undergoing apoptosis due to induction of HFE, and some of it were already dead which were dyed in red (Figure 4a). MTS assay further confirmed that the proliferation rate of HeLa cells cultured with SFH films was much lower than SF films (Figure 4b). And the proliferation rate of HeLa cells cultured with SFH films for 5 days was lower than HeLa cells cultured with SFH films for 3 days. Therefore, the MTS assay results indicated that SFH films keep releasing HFE to cell culture, causing proliferation rate of HeLa cells lower with increasing culture time.

3.3 | Cell apoptosis analysis

Apoptosis is a crucial physiological process that is considered as the preferred way to annihilate cancer cells (Ban et al., 2009; Ren et al., 2015). To elucidate whether the HeLa cells death induced by HFE occurred through apoptosis induction, we performed the apoptotic characterization in SFH-treated cells by Annexin V-FITC and PI double staining and flow cytometry analysis, and the percentage of apoptotic cells was calculated from the ratio of the sum of early plus late apoptotic cells at 1, 3, and 5 days of exposure, respectively.

As shown in Figure 5, no apoptotic nuclei were observed in the control, indicating that 99.9% of HeLa cells were in normal condition. After HeLa cells cultured with SFH films for 1 day, the percentage of early apoptotic cells increased to 3.59% in contrast with control cells (0.00%). Furthermore, after cultured with SFH films for 3 and 5 days, the percentage of apoptotic cells increases to 6.87% and 8.12% compared to the control, respectively. This means that SFH films continually released HFE to cell culture, leading more cells underwent apoptosis, the results were consistent with the MTS assay results.

3.4 | Cellcycle analysis

In addition, we characterized the effect of SFH films on cellcycle distribution of viable cells by FCM of PI-stained cells. The results demonstrated shifts in cellcycle distributions of viable cells after treatment (Figure 6). For the control, cellcycle distributions were $25.41 \pm 1.71\%$ in G1, $43.87 \pm 1.78\%$ in S, and $30.73 \pm 2.26\%$ in G2. After treatment with SFH, there was a highly significant shift in cellcycle distribution to $39.96 \pm 0.80\%$ (G1), $40.98 \pm 2.72\%$ (S), and $19.06 \pm 1.93\%$ (G2). These results indicated that SFH arrested HeLa cellcycle progression at G1 phase, resulting in the growth inhibition of the cancer cells. These results are in strong agreement with the cell apoptosis analysis, and SFH was shown to induce apoptosis in HeLa cells.

In our study, we used SF as a drug carrier to load HFE. The experiments of *in vitro* cytocompatibility, cell apoptosis assay and cell cycle analysis confirmed the apoptosis of HeLa cells resulted from HFE loaded silk fibroin-based films. Thus, SFH films may potentially be functionalized with cell-homing peptides for selective killing of cancer cells (Naveen et al., 2013; Sreeram, Shoba, Gopal, Andrew, & Mao, 2010)

4 | CONCLUSION

In summary, we have successfully prepared SF films loaded with HFE. SF was used as a drug carrier for HFE delivery for the first time. SEM and AFM confirmed that SFH films were prepared. MTS assay suggested that the proliferation rate of HeLa cells cultured with SFH films was lower than on SF films, agreeing with the results of fluorescence micrographs. Apoptosis assay further confirmed that a part of cells cultured with SFH films were undergoing apoptosis as the rate of apoptosis increased with time. Cellcycle analysis showed that SFH films arrested HeLa cellcycle progression at G1 phase, causing the growth inhibition of the cancer cells. It is in strong agreement with the cell apoptosis analysis. Hence, we expected that SFH films might be a promising candidate material for cancer therapy.

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