Electrospun Micelles/Drug-Loaded Nanofibers for Time-Programmed Multi-Agent Release[a]

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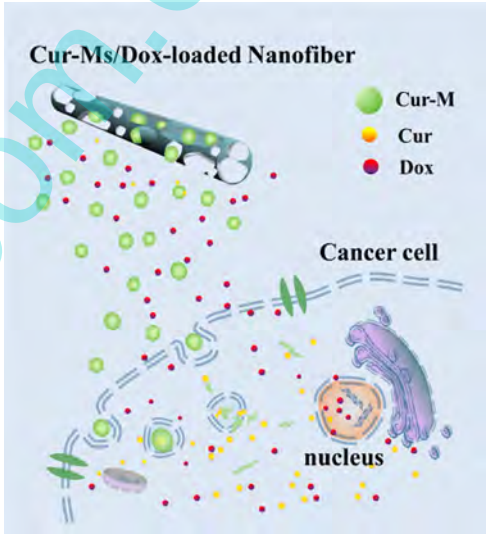
Combined therapy with drugs of different therapeutic effects is an effective way in the treatment of diseases and damaged tissues or organs. However, how to precisely control the release order, dose, and time of the drugs using vehicles is still a challenging task. In this work, for the first time, a study to develop a nanoscale multi-drug delivery system based on polymer micelle-enriched electrospun nanofibers is presented. The multi-drug delivery system is achieved, first, by the fabrication of hydrophobic curcumin encapsulated micelles assembled from biodegradable mPEG-PCL copolymer and, second, by the blending of the micelle powder with hydrophilic doxorubicin in polyvinyl alcohol solution, followed by simply electrospinning this combination. Due to the different domains of the two drugs within the nanofibers, the release behaviors show a time-programmed release, and can be temporally and spatially regulated. In vitro tumor cell inhibition assay indicates that the delivery system possesses great potential in cancer chemotherapy.



1. Introduction

The combination therapy of two or multiple drugs is a procedure clinically common in malignant disease treatment, and has led to significant increases in survival and quality of life due to the potential for additive or synergistic effects.[1,2] However, the potent toxic drug combination can damage normal cells, resulting in poor patient compliance since one critical constraint on delivering appropriate combinations of factors is a lack of delivery vehicles that allow for a localized and controlled delivery of these drugs.[3] To enhance the efficacy of drugs and attenuate their associated side effects, multi-agent delivery system, which contains dual agents with different therapeutic effects or biological functions, is gained more and more attention in recent years due to its unique advantages in combination therapies[4–7] or tissues regeneration and engineering.[3]

Currently, some efforts have been made to develop well-organized multi-release systems in a single formulation, of which, the release order, timing, dose, and duration for individual drugs need to be controlled precisely. So far, polymer scaffold,[3] hydrogel,[8,9] polymeric micelles,[10,11] hydrogel/polymer micelle composite,[12] "beads-embedded fibers",[13–15] and stimuli-responsive materials[16] have

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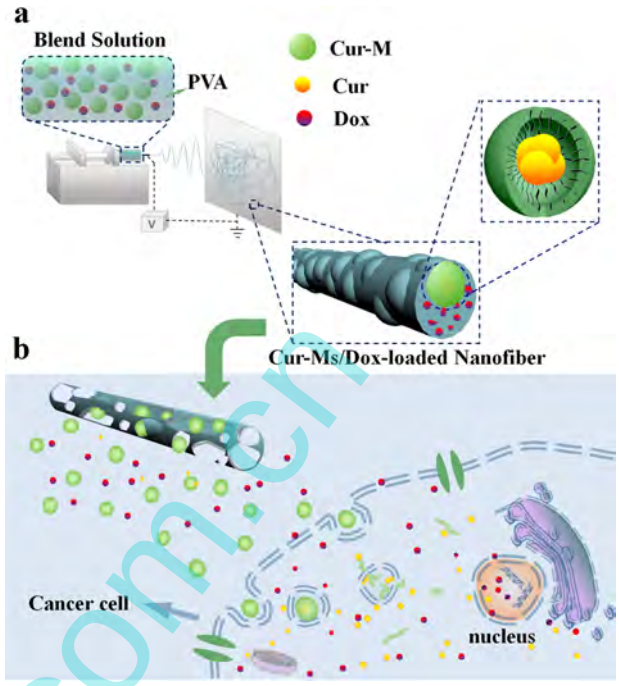
been employed to achieve this goal. In some degree, these release systems enable a temporally and spatially regulated release manner of dual drugs. Furthermore, time-programmed release, such as pulsatile release,[17,18] time-controlled explosion,[19] sigmoidal release[20] is a critical issue to improve the delivery effect. However, in practical application of the systems, there still exist many problems. For example, in the treatment of infection and pain after surgical procedure, the release of two or more different drugs cannot be controlled exactly at the proper time and in appropriate doses.[9,12] Additionally, in cancer therapy it is impossible only by the intravenous administration of drugs or multi-agent-loaded nanoparticles to achieve their sufficiently high concentration and long retention period in the tumor tissue because the drugs with small molecular size or the ones leaked and released from nanoparticles are easy to rapidly excrete from the blood circulation.[21,22]

To establish a versatile and general methodology for improving the efficiency of combination therapy of multiple agents, in this study we describe the first study to develop a novel nanoscale multi-drug delivery system with time-programmed release based on polymer micelle-enriched electrospun nanofibers. Electrospun nanofibers with a high surface area to volume ratio have received much attention because of their potential applications for biomedical devices, tissue engineering scaffolds,[23] wound dressing,[24] and drug delivery carriers,[25] mainly due to the structural similarity to the tissue extracellular matrix and the processing availability to a variety of materials.[26] Micelles with a core-shell architecture, which are formed through self-assembly of amphiphilic block copolymers, are being widely investigated as nanocarriers to increase the solubility and decrease the toxicity of hydrophobic drugs, and have found particular utility in the delivery of cancer therapeutics as one of the most promising platforms in cancer therapy.[27,28] However, several characteristics limit the efficiency of modern micelle systems including inadequate drug loading capacity, poor stability in blood circulation, and insufficient binding and uptake by cells.[28]

In achieving a more stable micelle system and maintaining micelle advantages as a vehicle in drug delivery system, these micelles loaded with one class of model anticancer drug (Curcumin-loaded micelles; Cur-Ms), hydrophobic curcumin (Cur), can be immobilized in polymer nanofibers in this work as shown in Scheme 1. At the same time, another model drug, hydrophilic doxorubicin hydrochloride (Dox), is also trapped within the fiber matrix. This model system, Cur-Ms/Dox-loaded nanofibers (Cur-Ms/Dox-nanofibers), can be acquired by electrospinning the polyvinyl alcohol (PVA) water solution containing both Cur-Ms and Dox. The polymeric micelles is fabricated from the biodegradable and amphiphilic monomethoxy poly-(ethylene glycol)-block-poly(ε-caprolactone) (mPEG-PCL) copolymer.[29] The good stability of the micelles can be



Scheme 1. Schematic illustrations of a) the fabrication of the nanoscale multi-agent delivery system (Cur-Ms/Dox-loaded nanofibers) via a simple electrospinning process and b) the time-programmed release of Cur-Mss and Dox from the nanofibers, after that, both the released micelles and Dox are delivered to cancer cells, in which the intracellular release of Cur is achieved.

achieved by immobilizing them in nanofiber matrix, and thus the drug leaking generally occurring in modern micelle systems can be effectively inhibited. Since the PVA is water-soluble, both Dox and Cur-loaded micelles can be firstly released from the nanofibers in an aqueous environment. Later, free Cur can be gradually released from the released polymeric micelles. Thus, this model system can allow multiple drugs to be safely embedded within the fiber matrix and enable the release of each drug to be independently controlled. In cancer therapy, the released Cur-Ms and Dox can be firstly uptaken by cancer cells, and then Cur can be intracellular released, suggesting that the two anticancer drugs are combined and programmed released to inhibit the growth of tumor cells.

2. Experimental Section

2.1. Materials

PVA (hydrolysis degree: 1788) was purchased from Kelong Chemicals (Chengdu, China). Poly(ε-caprolactone) (PCL, $\overline{M}_w \sim \approx 100$ kDa) and amphiphilic mono-methoxy poly(ethylene glycol)-block-poly(ε-caprolactone) (mPEG-PCL) copolymers were synthesized as previously described.[29] Curcumin was purchased from Chengdu

labooo Company (Chengdu, China). Doxorubicin hydrochloride (Dox) was purchased from Zhejiang Hisun Pharmaceutical Co. Ltd. (China). 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI), was purchased from Sigma chemicals (St. Louis, MO, USA). All other chemicals with reagent grade were obtained from commercial sources and used without further purification. Deionized (DI) water was used in all experiments.

2.2. Preparation of the Blank and Curcumin-Loaded Polymeric Micelles

The blank and Curcumin-loaded micelles (Cur-Ms) were fabricated by a solvent evaporation method. Briefly, to prepare blank micelles, 0.1 g of mPEG-CL copolymer was dissolved in 10 mL tetrahydrofuran (THF), then the copolymer solution was added dropwise using a disposable syringe (21 gauge) into 10 mL DI water under high-speed stirring. The mixed solution was then stirred moderately for 4 h at room temperature to evaporate the THF completely and form polymeric micelle solution. For long-time preservation, the micelle solution was freeze-dried into micelle powder. To prepare the Cur-Ms, similar procedure as previously described was carried out in darkness. The difference is that 0.01 g Curcumin and 0.1 g mPEG-CL copolymer were dissolved in the 10 mL THF at the same time. Then, the Cur-Ms solution were transferred into dialysis bag (MWCO: 1000) with DI water to remove the unloaded Curcumin before freeze-drying. Drug loading content (LC) and encapsulation efficiency (EE) were measured with UV-vis spectrophotometer (UV-2550, Shimadzu, Japan) and calculated as previous report.[30]

2.3. Preparation of the Blend Solution of the PVA and Cur-Ms/Dox

The blend solutions of Cur-Ms and PVA with Cur-Ms: PVA weight ratios of 1:4, 1:2, 1:1 were obtained by following procedures. Firstly, a 20 w/v% PVA solution was obtained by dissolving 0.5 g PVA into 2.5 mL DI water at 85 °C and stirred for about 4 h until complete dissolution. After the PVA solution was cooled down, another 2.5 mL DI water with calculated amount of Cur-M powder (0.125 g, 0.25 g, 0.5 g, respectively) was added into the PVA solution, in darkness, to achieve the desired Cur-Ms: PVA weight ratio and to keep the final concentration of PVA constant at 10 w/v%. The mixture was vigorously stirred at room temperature until a homogeneous viscous blend solution was obtained. The blend solution of 10 w/v% PVA and 0.4 wt% Dox, and the blend solution of 10 w/v% PVA, 0.4 wt% Dox, and Cur-Ms with Cur-Ms: PVA weight ratio of 1:2 was also prepared as mentioned above. A 10 w/v% pure PVA solution was as a blank control.

2.4. Electrospinning of the Blend Solution

As shown in Scheme 1, the homogeneous blend solution was loaded in a 5 mL plastic syringe connected to a stainless needle (inner diameter: 0.7 mm). The syringe was fixed horizontally on a microinjection pump (LongerPump, LSP02-1B, China). The collector, a grounded plate coated by aluminum foil, was placed 14 cm away from the tip of the needle and a potential was applied between the needle and the collector. All the blend solutions and pure PVA solution were electrospun at 14 kV under a flow rate of 0.4–1 mL h^{-1}. All the electrospun fibers were collected in darkness at room temperature. Using different blend solutions, Cur-Ms-loaded nanofibers (Cur-Ms-nanofiber) (with different weight ratio of Cur-Ms/PVA), Dox-loaded nanofibers (Dox-nanofibers) and Cur-Ms/Dox-loaded nanofibers (Cur-Ms/Dox-nanofibers) were obtained by the electrospinning.

2.5. Characterization of Cur-Ms and Micelle-Loaded Nanofibers

Atomic force microscopy (AFM) (CSPM5000, Being, China) and transmission electron microscope (TEM) (JEM-2100F, JEOL Ltd., Japan) were employed to observe the morphology of the Cur-Ms. The AFM sample was prepared by placing a drop of micelle solution (1 mg mL^{-1}, filtered through a 450 nm syringe filter) on a silicon wafer, and dried at room temperature before AFM observation. Tapping-mode AFM was utilized to scan the sample. The TEM sample was prepared by placing a drop of micelle solution (1 mg mL^{-1}) on a carbon film-coated copper grid and stained by phosphotungstic acid aqueous solution (0.5 wt%). Cur-Ms released from the nanofibers were also observed by AFM and TEM. Firstly, 3 g Cur-Ms-nanofibers with Cur-Ms: PVA weight ratio of 1:2 were dissolved in 3 mL DI water for at least 1 h at room temperature. Then the solution was filtered through a 450 nm syringe filter. The filtered solution was employed to prepare the AFM sample and the TEM sample as described above.

Dynamic light scattering (DLS) measurements (Malvern Zetasizer Nano-ZS90 apparatus) were performed in aqueous solution to determine the particle size variation of Cur-Ms before encapsulation and after releasing from the fibers.

The morphologies of all of the nanofibers were observed by a field emission scanning electron microscope (FE-SEM) (JSM-7001F, JEOL Ltd. Japan). Before observation, all of the samples were sputtered with platinum film. Images of three randomly selected areas were captured and analyzed by ImageJ software (1.46 h, NIH, USA) to determine the diameter of nanofibers.

Cur-Ms/Dox-nanofibers with Cur-M:PVA weight ratio of 1:2 were collected on glass slides and stored in darkness. Confocal laser scanning microscopy (CLSM) (Olympus FV1000) was applied to visualize the Cur-Ms inside the nanofibers. Cur and Dox were both excited at 488 nm. The emission wavelengths of Cur and Dox are 520 and 595 nm, respectively.

2.6. Pretreatment of All Nanofiber Samples

Before drug release study and cell culture, all fiber samples were pretreated as follows. First of all, the dried electrospun nanofiber meshes were cut into small round flakes with an average diameter of 1 cm (shown in Figure 4a). Each round flake was exactly measured with 1.5 mg weight. Then the small round flake was dipped in PCL solution (4 w/v%, PCL in dichloromethane) for about 5 s and dried in air.

2.7. Water Contact Angle

The hydrophilicity of pure PVA nanofibers, Cur-Ms-nanofibers and Cur-Ms/Dox-nanofibers were characterized by water contact angle (WCA) measured by a sessile drop method with the contact angle

equipment (DSA 100, KRUSS, Germany) at room temperature. Both WCA values of the right side and the left side of the DI water droplet were measured, and an average value is used.

2.8. In Vitro ReleaseModel Drugs

Cur-Ms/Dox-nanofiber meshes (Cur-M:PVA weight ratio = 1:2) and Dox-nanofiber meshes were chosen to study the time-programmed release behavior of Cur and Dox. Three flakes of the pretreated sample (4.5 mg) coated with PCL were placed in the dialysis bags (MWCO: 1000) and incubated in 15 mL of phosphate buffered saline (PBS, pH 7.4) or acetate buffered solution (ABS, pH 5.0), respectively in test tubes. Tween-80 was selected as the emulsifier on the basis of maximum in vitro bioaccessibility of curcumin.[31] The tubes were kept in a thermostated incubator that was maintained at 37 °C and with a shaking speed at 100 cycles min^{-1}. At pre-set interval, 1 mL of release medium outside the dialysis bag was collected and 1 mL fresh PBS or ABS was added back. The concentration of released Cur and Dox was determined by a fluorescence spectrophotometer (F-7000, Hitach, Japan).[32] The cumulative amount of the released Cur and Dox was calculated, and the percentages of these two drugs released were plotted against time.

To further study the release behavior of Cur, Cur-M solution, and Cur-Ms-nanofiber meshes (2:1 PVA/Cur-M weight ratio) were chosen. Ten milligrams freeze-dried Cur-M powder was re-suspended in 10 mL PBS or ABS. Two milliliters of the Cur-M re-suspension was transferred into dialysis bags (MWCO: 1000) and immersed into 15 mL PBS or ABS. Samples (4.5 mg) coated with PCL were placed inside or outside the dialysis bags (MWCO: 1000) and incubated in 15 mL PBS or ABS. The rest procedure of the release experiment was carried out as mentioned above, except that the concentration of released Cur was measured by high performance liquid chromatography (HPLC, Agilent 1260 Infinity) and the collected samples were diluted with acetonitrile.[33]

2.9. Cell Culture

Osteoblasts (OB) from neonatal rat's mandibular belonged to normal cell line were grown in α-modified essential medium (α-MEM) (HyClone, USA) with 10% newborn calf serum (NBCS, Gibco, USA). The cancer cells (HeLa) as a gift from Sichuan University (China) were grown in RPMI medium 1640 (HyClone, USA) with 10% NBCS. All the cells were cultured at 37 °C and 5% CO_2 under fully humidified conditions.

2.10. Cytotoxicity Assay

The cytotoxicity of blank micelles and pure PVA nanofiber meshes were studied using OBs and HeLa cells by Alamar blue assays. Five milligrams blank micelle powder was re-suspended in 1 mL cell culture medium to obtain a 5 mg mL^{-1} blank micelle sample solution. The pure PVA nanofiber meshes were pretreated as described above. All of the samples were sterilized by ultraviolet irradiation through UV lamps before cultured with cells. For blank micelles, OB or HeLa cells were seeded at a density of 2×10^4 cells $well^{-1}$ into 48-well plates with α-MEM with 10% NBCS or RPMI 1640 with 10% NBCS, respectively. After 24 h, the medium was replaced with 0.9 mL of fresh medium and 0.1 mL pre-prepared blank micelle sample solution was added to incubate with the cells. Then, the cells were incubated for up to 7 days. After the first day, the medium containing blank micelles was replaced by the fresh medium. In the rest 6 days, the medium was refreshed completely every other day.

For pure PVA nanofibers, cells were seeded at a density of 2×10^4 cells $well^{-1}$ directly on the nanofiber flakes in 48-well plates and were cultured for up to 7 days. The cells were also cultured on the tissue culture plates (TCPs) without any samples for 7 days as control. The culture medium was refreshed every other d. At time points of 1, 3, 5, and 7 days, medium was carefully removed from the wells and cells were washed with PBS twice, then 250 mL Alamar blue solutions (10% Alamar blue (Biosource, Nivelles, Belgium), 80% media 199 (Gibcos, USA) and 10% FBS; v/v) were added into each well and incubated for further 3 h at 37 °C, 5% CO_2. Wells without cells were used as the blank controls. Then, 200 μL of reduced Alamar blue solution was pipetted into 96-well plate (Sigma) and read at 570 nm (excitation)/600 nm (emission) in a ELISA microplate reader (Molecular Devices, Sunnyvale, CA). Additionally, the morphology of OBs and HeLa cells grown on the surface of nanofiber flakes after 7 days was evaluated by SEM (FEI, Quanta 200, Philips, Netherlands). For SEM observation, the samples were washed twice with PBS and fixed with 2.5% glutaraldehyde overnight at 4 °C. After that, the specimens were further dehydrated through a series of graded alcohol solutions and dried overnight. The dried samples were finally sputter coated with gold and observed under the SEM at an accelerating voltage of 20.0 kV.

2.11. In Vitro Cancer cell Growth Inhibition Assay

Viability of HeLa cancer cells treated with different samples for 1, 3, 5, 7 days is evaluated in two ways as shown in Figure 5. Way 1 (Figure 5a) is to culture cells directly onto the nanofiber meshes including pure PVA nanofibers, Cur-Ms/Doxnanofibers (Cur-Ms: PVA weight ratio = 1:2, the content of Dox = 0.4 wt%), Cur-Ms-nanofibers (Cur-Ms:PVA weight ratio = 1:2) and Dox-nanofibers (the content of Dox = 0.4 wt%). The procedure of cell culturing is the same as mentioned in Cytotoxicity assay. The morphology of HeLa cells cultured on pure PVA nanofibers and Cur-Ms/Dox-nanofibers after 3 days were observed by SEM. Cells cultured on pure PVA nanofibers and on TCPs without samples were as controls.

Way 2 (Figure 5b) is to culture cells on TCPs, and after the cells already attached and spread well on TCPs, the nanofiber meshes were added and fixed between two glass rings. The groups of pure PVA nanofibers, free drugs (Cur and Dox), micelles (blank micelles and Cur-Ms) and cells on TCPs without any samples were used as control. The same culture procedurs as way 1 were carried out, except that cells were seeded on TCPs and samples were added 24 h later. After the first day, the medium in all groups was replaced by the fresh medium without any drugs. In the rest 6 days, the medium was refreshed completely every other day. The corresponding cell micrographs were obtained by fluorescence microscope (Olympus, CKX41). DAPI were used to stain the nuclei of cells.

Cur and Cur-Ms were added with an equivalent Cur dosage of 30 μg mL^{-1} with that loaded in Cur-Ms-nanofibes and Cur-Ms/

Dox-nanofibers. Dox was added with the same dosage of 6 $\mu g\ mL^{-1}$ as that loaded in Cur-Ms/Dox-nanofibers and Dox-nanofibers. All of the samples were applied to the following experiment.

2.12. Determination of the Internalization

CLSM (Olympus, FV1000) was performed to observe the internalization of Cur-Ms and Dox. Firstly HeLa cells were incubated with drug-loaded fiber groups (Cur-Ms/Dox-nanofibers and Cur-Ms-nanofibers), free drugs (Cur and Dox), and Cur-Ms in way 2. After culturing 4 and 12 h, the medium was removed and each well was rinsed with PBS, then cells were fixed with 2.5% glutaraldehyde and the nuclei of cells were stained by DAPI. Cur and Dox were both excited at 488 nm. The emission wavelengths of Cur and Dox are 520 and 595 nm, respectively.

2.13. Statistics Analysis

All experiments were performed in triplicate specimens and the results were showed as mean ± standard deviation. Single factorial analysis of variance (ANOVA) was performed to determine statistical significance of the data.

3. Results and Discussion

3.1. Encapsulation of Cur-Ms and Dox in PVA Nanofibers

To realize the time-programmed multi-agent release from a single formulation, the successful encapsulation of two distinct compounds at separated domains in electrospun nanofibers is a key factor. In this work, the micelles/drug-loaded nanofibers are achieved by, first, the successful fabrication of Cur encapsulated micelles (Cur loading content: 6.16%; encapsulation efficiency: 67.76%) with an average size of 139 nm assembled from mPEG-PCL copolymer, second, the blending of the micelles in freeze-dried powder with Dox in PVA solution, followed by electrospinning this combination. By changing the concentration of the micelles in PVA solution with concentration of 10 w/v %, we can obtain a series of micelles-loaded nanofibers. To confirm whether the micelles were successfully loaded in the nanofibers, we firstly investigate the structure of the nanofibers using a FE-SEM. From the SEM images in Figure 1, we can find that the pure PVA nanofibers are uniform in diameter (270 ± 37 nm) and smooth in appearance without "spindle-knots" (Figure 1a), however, after introduced Cur-Ms, the surface of the nanofibers (Cur-Ms-nanofibers) becomes rough and some spindle-knots can be found as shown in Figure 1b and b′–d and d′. With the increasing of the weight ratio of Cur-M: PVA from 1:4 to 1:1, the average diameter of the nanofibers increased from 220 ± 50 to 314 ± 38 nm, and for spindle-knots increased from 377 ± 44 to 562 ± 26 nm. Although, unlike silica nanospheres,[34] the aggregates of polymeric micelles cannot be reliably identified in the nanofibers because of the absence of contrast between both polymers, the variations of fibers in appearance and average diameters between pure PVA nanofibers and micelles-loaded nanofibers also give evidence that, at least, the introduction of micelles lead to the variation of the fibers' morphology. TEM was applied to further demonstrate the encapsulation of micelles as shown in Figure S1, Supporting Information. From Figure S1, Supporting Information, spindle-knots can also be found along the fibers, which is agree with the SEM results, furthermore, the regions of the "spindle-knots" are darker than the regions nearby, this may be owing to that the copolymer has relatively much higher crystallinity than PVA, which maybe an direct evidence that micelles were encapsulated inside the fibers in some degree.

CLSM was further performed to directly visualize the distribution of both encapsulated Cur-Ms and Dox within the Cur-Ms/Dox-nanofibers with the weight ratio of Cur-M: PVA of 1:2 (Figure 2). The encapsulated Cur-Ms and Dox are not visible under a bright-field mode (Figure 2a), but the continuous nanofibers with spindle-knots can be found. The Cur-Ms emit green light (Figure 2b) and Dox molecules fluoresce red (Figure 2c) because of the autofluorescence of both Cur and Dox. Also, we can find that the Cur-Ms aggregates distribute discontinuously along the fiber axis, but it is difficult to distinguish a single micelle limited to the resolution of CLSM.[35] Dox within the nanofibers shows a relative more continuous distribution along the nanofibers, suggesting that Dox is dispersed more uniformly in fiber matrix. Figure 2d is an overlaid image, indicating the successful encapsulation of Cur-Ms and Dox in distinct domains within the electrospun nanofibers.

Water-treated Cur-Ms-nanofibers were also characterized under CLSM (Figure S2, Supporting Information). The nanofibers were firstly collected on a glass slide, then a drop of DI water was add onto the slide, and later, the sample was observed with CLSM. From the bright-field image (Figure S2a, Supporting Information), no fiber-like structure can be found, however, a large number of green fluorescent spots are observed in the fluorescence image (Figure S1b, Supporting Information). This result indicates that the structure of nanofibers is entirely destroyed since the PVA matrix of the nanofiber is dissolved in water and the aggregates of Cur-Ms arranged in the nanofibers are exposed. It is also in good agreement with the results of Figure 1 and 2 that the Cur-Ms were successfully encapsulated in the nanofibers.

To further investigate whether the structure of polymeric micelles is damaged by the electrospinning process, Cur-Ms released from the Cur-Ms-nanofibers were gained and characterized by TEM, AFM, and DLS measurement, respectively. From Figure 3, we can find that the morphologies and size distribution of the released micelles

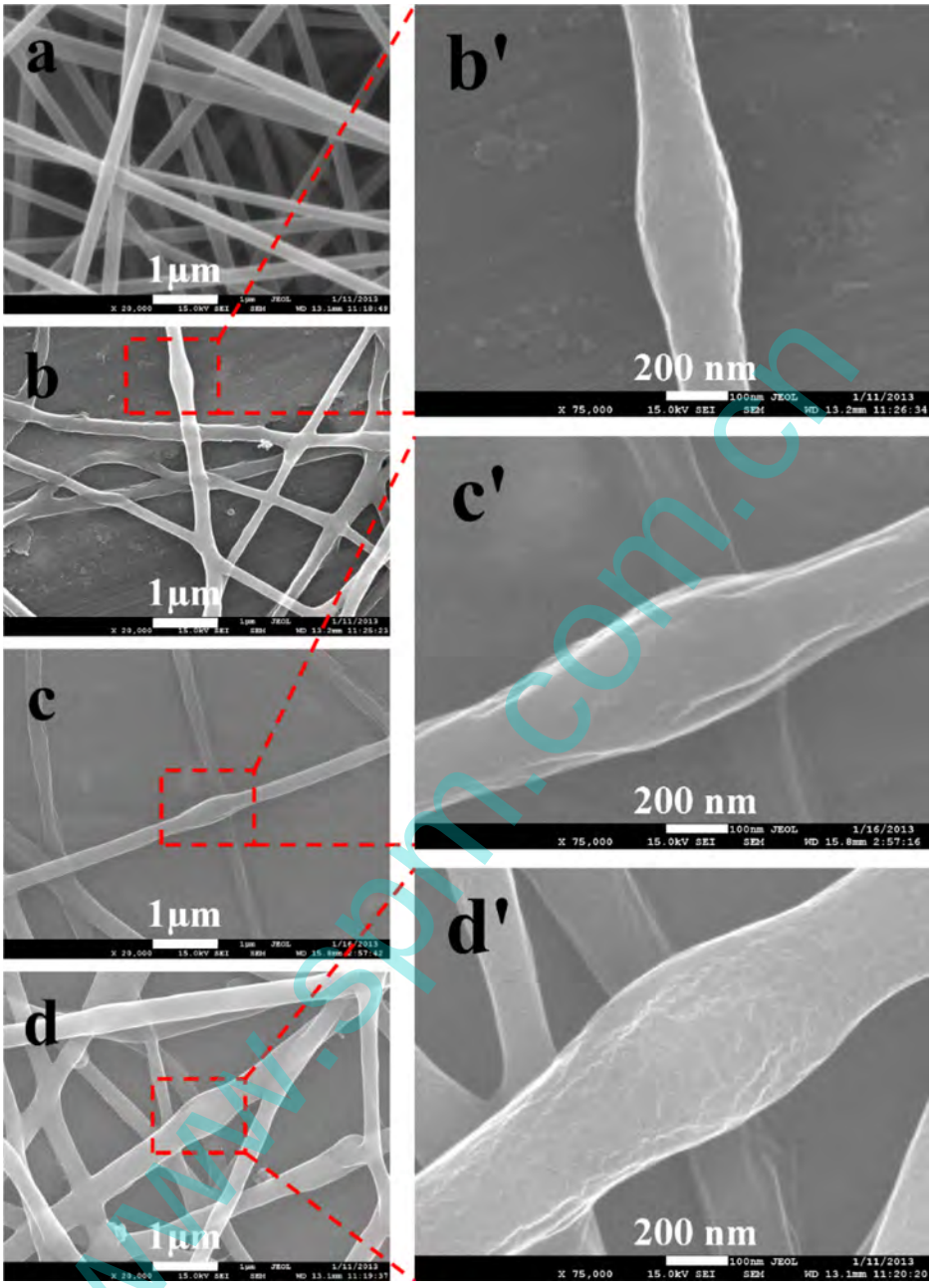


Figure 1. SEM images of the electrospun Cur-Ms-nanofibers with different Cur-Ms: PVA weight ratios: a) pure PVA nanofibers; b,b′) 1:4; c,c′) 1:2; d,d′) 1:1. The concentration of PVA was constant to 10 w/v% and Dox was not loaded in these samples.

are almost identical compared with the original ones, indicating that the electrospinning could not damage the structure of the micelles. Also, it is very critical to maintain a good stability potentially in blood circulation and prevent from the leaking of drug in advance from the single micelle system since they are immobilized in nanofiber matrix. The average diameter of the released micelles turns slightly larger than that of original ones, which may be due to the wrapping of micelles by residual PVA macromolecular chains.

3.2. Time-Programmed Multi-Agent Release

As multiple drugs were incorporated in the nanoscale drug delivery system, within which Cur located in the cores of micelles and Dox distributed in the fiber matrix, the release

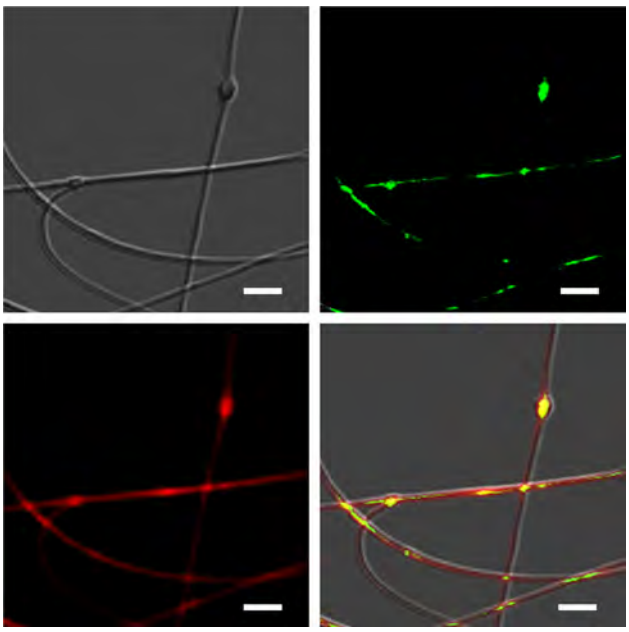


Figure 2. CLSM images of the electrospun Cur-Ms/Dox-nanofibers (Cur-M: PVA weight ratio = 1:2, the content of Dox = 0.4 wt%): a) Bright field; b) emission of the Cur-Ms; c) emission of the Dox and d) an overlaid representation of all the components. Scale bar = 5 μm.

behaviors of both Cur and Dox from this system were investigated. To prevent rapid dissolution of PVA, a pretreatment of the nanofibers coated by poly(ε-caprolactone) (PCL) was carried out before the release study, as shown in Figure 4a. From the SEM images, we can find that the morphology of the nanofibers is almost not influenced by the coating of PCL. Figure 4b shows the time-programmed release behavior of Cur and Dox from the Cur-Ms/Dox-nanofibers with weight ratio of Cur-M: PVA of 1:2 at different pH values during 14 days. The single Dox-loaded nanofibers (Dox-nanofibers) were used as a control to investigate whether the encapsulation of Cur-Ms influences the release of Dox. It is noteworthy that all the samples were put into the dialysis bags and immersed in ABS at pH 5.0 and PBS at pH 7.4 respectively to ensure only drugs being collected. Figure 4b revealed that after 14 days the cumulative release of Dox was 98.27 ± 9.20% at pH 5.0 and 96.18 ± 2.11% at pH 7.4, while the total release of Cur was 43.33 ± 4.23% at pH 5.0 and 31.11 ± 3.99% at pH 7.4. Furthermore, in Figure 4b, it is easy to find that in the initial 12 h, the release of Dox was significantly faster than that of Cur under the same conditions. After 12 h, the release of Cur began to level off and the release of Dox started to slow down. The release profile of Dox from the Cur-Ms/Dox-nanofibers is matched with that from the Dox-nanofibers, indicating that the release of Dox is independent. The initial burst release of Dox is attributed to that the hydrophilic Dox is distributed in the PVA matrix and the dissolving of PVA. However, since the Cur is encapsulated in polymeric micelles that are immobilized within the PVA matrix, it can be released by the diffusion of Cur and the de-assembly of the released micelles after the dissolving of PVA, therefore the release of cur is slow and sustained. This result demonstrates that the release of Dox and Cur is indeed a time-programmed procedure, which is mainly owing to their different locations in the nanofibers.

To further understand the release mechanism of Cur from nanofibers, the Cur-Ms-nanofibers inside and outside dialysis bags and Cur-Ms inside dialysis bags were

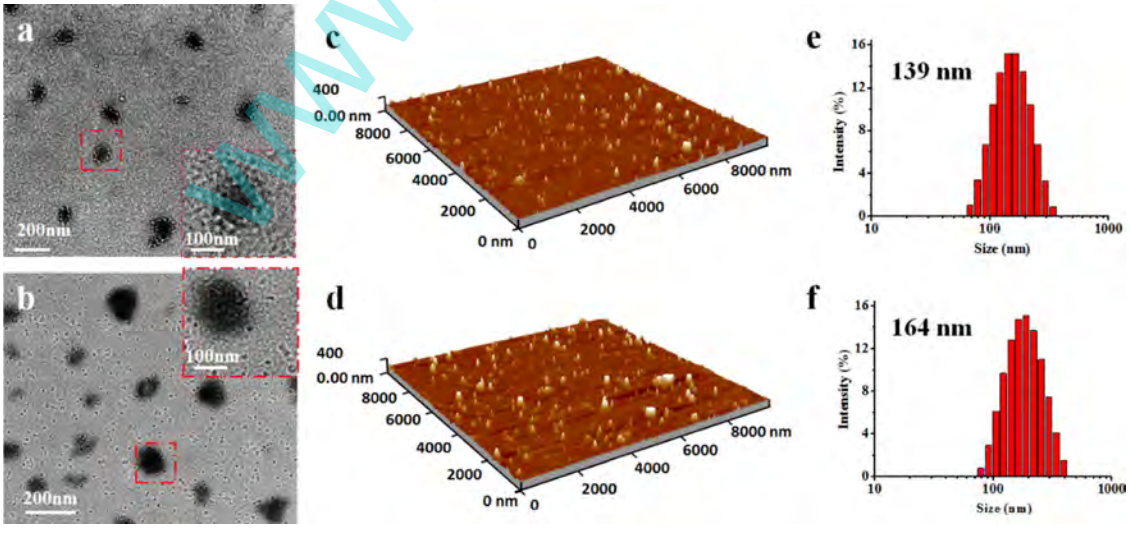


Figure 3. a,b) TEM images, c,d) AFM images, and e,f) DLS measurement of the Cur-Mss a,c,e) before loaded in the electrospun nanofibers and b,d,f) after released from the nanofibers.

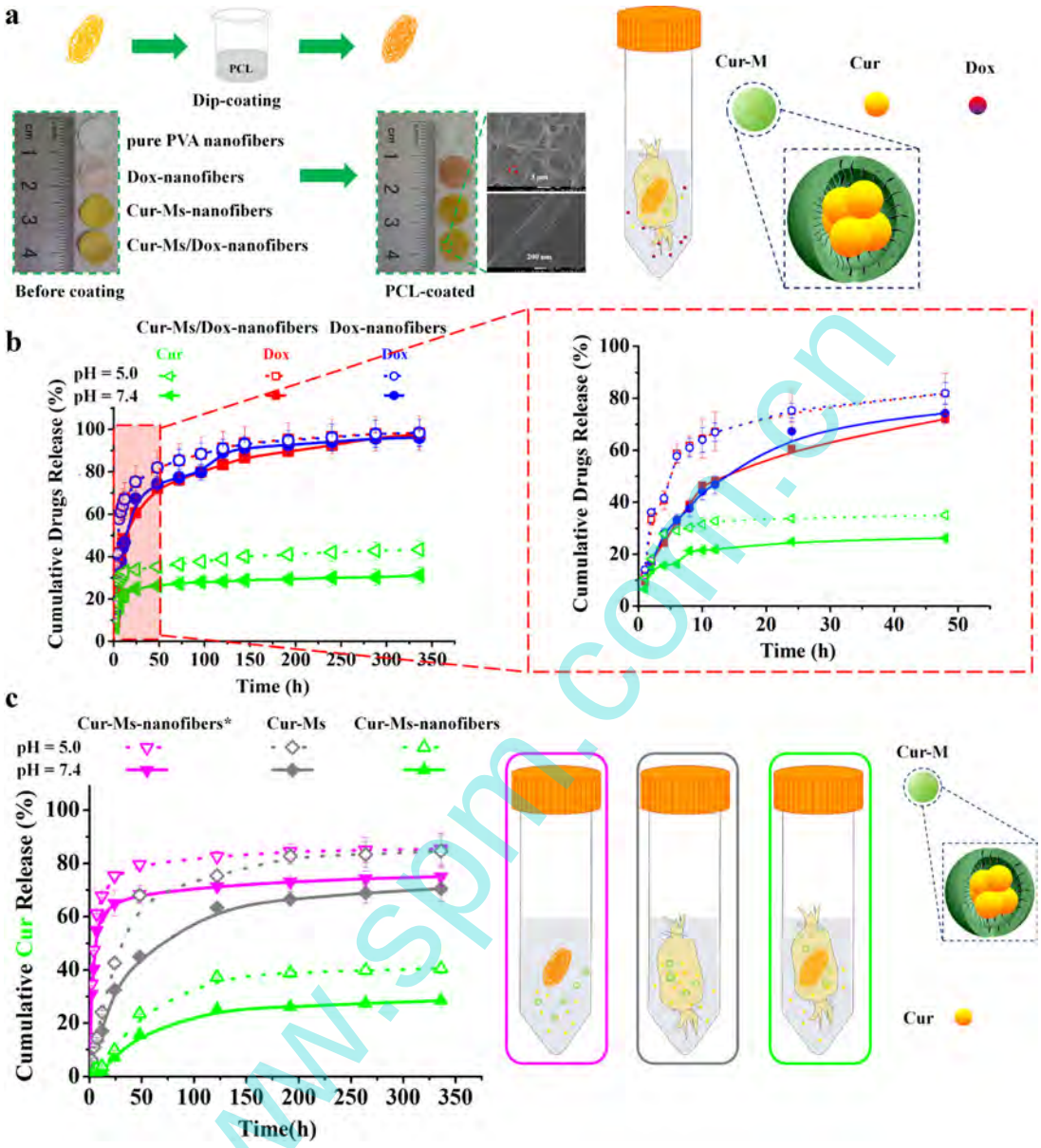


Figure 4. Release behaviors of Cur and Dox from the electrospun nanofibers. a) The schematic illustration of the nanofibers treated by PCL solution via a "Dip-coating" method, and these photos showing the appearance of the nanofiber meshes before and after the treatment. b) The cumulative release profile of Cur and Dox from Cur-Ms/Dox-nanofibers and Dox from the Dox-nanofibers. Both the two samples were put in dialysis bags and immersed in PBS (pH 7.4) or ABS (pH 5.0). The dashed panel is a highly magnified view of the original release period within 50 h. c) The cumulative release profile of Cur from the Cur-loaded micelles and Cur-Ms-nanofibers inside and outside of dialysis bags in PBS (pH 7.4) or ABS (pH 5.0) (Cur-Ms-nanofibers* represents the samples outside dialysis bags).

employed to study the release behavior. The samples are placed in dialysis bags, only the free drug molecules released can be collected and the polymeric micelles cannot be obtained due to the pore size of dialysis bags, however, for samples placed outside dialysis bags, both the drug and the micelles are collected. As shown in Figure 4c, when samples were placed inside dialysis bags, the cumulative release of Cur from Cur-Ms reached 42.61 ± 1.14% at pH 5.0 and 32.53 ± 0.24% at pH 7.4 within the initial 24 h, however, the release of Cur from Cur-Ms-nanofibers got to only 9.81 ± 0.44% at pH 5.0 and 7.13 ± 0.77% at pH 7.4, indicating that the encapsulation of the Cur-Ms into nanofibers can resolve the problem of the drug leaking from the single micelle system.

When placed the Cur-Ms-nanofibers outside dialysis bags, a burst release of Cur was happened in the initial 24 h and the cumulative release percentage of Cur reached 75.31 ± 1.14% at pH 5.0 and 65.01 ± 4.83% at pH 7.4. Since

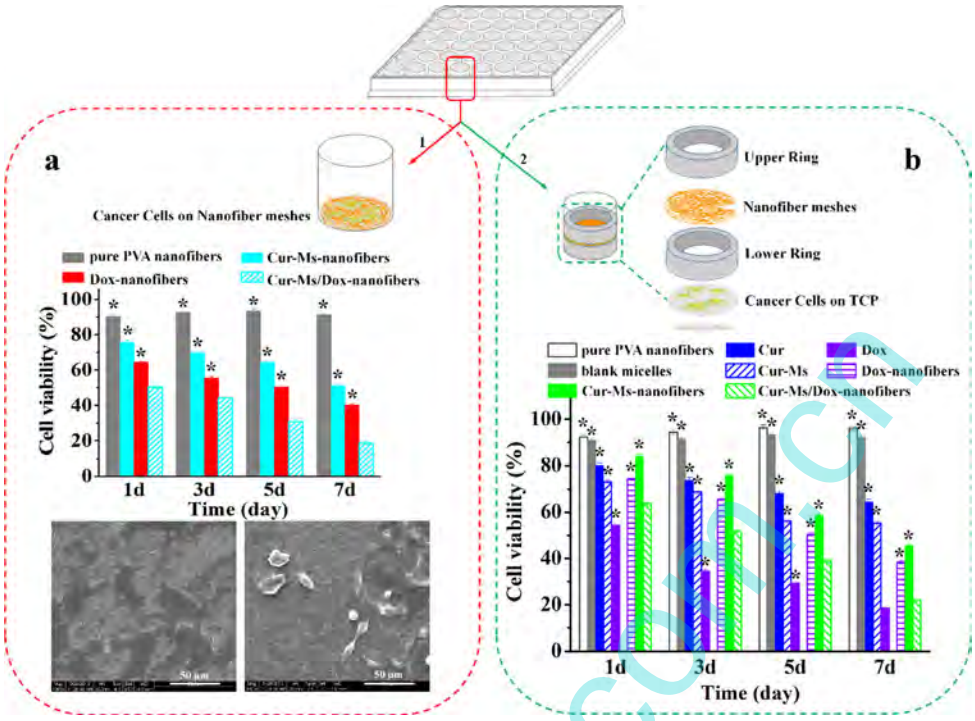


Figure 5. Viability of HeLa tumor cells treated with different samples for 1, 3, 5, 7 days, which is evaluated with two ways: a) Way 1 is to culture cells directly onto the nanofiber meshes. The histogram shows the viability, and SEM images show the morphology of HeLa cells on pure PVA nanofibers (left) and Cur-Ms/Dox-nanofibers (right) after 3 days culturing. b) Way 2 is to culture cells on TCPs, and after the cells already attached and spread well on TCPs, the nanofiber meshes were added and fixed between two glass rings, thus cells cultured did not attach on the nanofiber meshes, but Cur-Ms and Dox could still be released into the culture medium from the nanofibers. The histogram shows the viability of HeLa cells. *Stands for the data that have significant difference ($p < 0.05$, $n = 3$), as compared with Cur-Ms/Dox-nanofibers group at the same time point.

both the released Cur and Cur-Ms can be collected when the samples were put outside dialysis bags, the sharp increase in burst release of Cur was achieved in contrast to the burst release from the samples inside dialysis bags. This result is also confirmed by the fluorescence emission spectra of Cur release medium from the Cur-Ms-nanofibers placed inside and outside dialysis bags at 24 h (Figure S3, Supporting Information). Two peaks near 500 and 540 nm can be found in the spectrum of the Cur release medium from the Cur-Ms-nanofibers outside dialysis bags, while only one peak near 540 nm can be found from the one inside dialysis bags. According to previous reports,[36,37] the strong blue-shift occurred in the fluorescence emission spectra of the Cur release medium can be ascribed to the interaction between Cur molecules and polymeric micelles. Additionally, in contrast to the release of Cur from Cur-Ms-nanofibers inside dialysis bags, we can find that the Cur-Ms/Dox-nanofibers display a faster release in the initial 12 h and followed a sustained release, but the cumulative release percentage of Cur from both of the two groups are almost equivalent in the whole period. The reason can be ascribed to that the hydrophilicity of Cur-Ms/Dox-nanofibers increased with the introduction of hydrophilic Dox into Cur-Ms-nanofibers, which is also evidenced by the measurement of WCAs as shown in Figure S4, Supporting Information. The Cur-loaded micelles are hydrophobic. The WCAs of all these samples increases after treated with hydrophobic PCL polymer. Especially, after the introducing of Dox into Cur-Ms-nanofibers, the WCA cannot be measured, indicating that the material is fully hydrophilic. The difference of the hydrophilicity between the Cur-Ms/Dox-nanofibers and Cur-Ms-nanofibers leads to the different release speed of the loaded cur-Ms. In a word, in the multi-agent delivery system, the hydrophilic drug distributed in the water-soluble fiber matrix was released with the dissolving of the matrix, simultaneously, the loaded micelles containing hydrophobic drug within fiber matrix were also liberated, later, most of the hydrophobic drug encapsulated into the released micelles was gradually released.

3.3. Potential of Cur-Ms/Dox Loaded Nanofibers in Cancer Therapy

At first, the cytotoxicity of the materials was studied by culturing OBs and HeLa cancer cells on PCL-coated pure PVA nanofibers and with blank micelles. From Figure S5a,

Supporting Information, we can find that the percentage of OB and HeLa cells viability, quantified by the Alamar blue assays, are both more than 85%. Moreover, from the SEM images in Figure S5b and b′ and c and c′, Supporting Information for OB and HeLa cells cultured on PCL-coated pure PVA nanofibers after 7 days, we can observe that these cells were attached and spread well on the fiber meshes. The results demonstrate that both PCL-coated pure PVA nanofibers and blank micelles possess good cytocompatibility.

Later, we focus on investigating the potential application of Cur-Ms/Dox-nanofibers in cancer therapy as a model system. Since the nanofiber meshes were loaded with Cur and Dox, they may have a negative effect on cell adhesion[38] after cells seeded onto the fiber meshes. Thus, if cells are directly seeded on the nanofiber meshes, cell viability quantified by the Alamar blue assays could not truly reflect the inhibition effect of the samples because of the poor attachment of the cells. Considering this reason, the inhibition of the samples to cancer cells was performed in two ways as shown in Figure 5a,b. Way 1 in Figure 5a is to culture the HeLa cells directly on the Cur-Ms/Dox loaded nanofiber meshes as previous reports,[39,40] while way 2 in Figure 5b is to culture the cells not contacting the nanofiber meshes, which is achieved by, first, culturing the cells on TCPs, then fixing the nanofiber meshes between two glass rings to let Cur-Ms and Dox be released from the nanofibers into the culture medium. Since all of these samples were added after the cells already attached and spread well on TCPs, the viability of cells can reflect the inhibition effect of the samples more truly and accurately.

The histogram in Figure 5a shows the viability of HeLa cells cultured directly on Cur-Ms/Dox-nanofibers, Cur-Ms-nanofibers, Dox-nanofibers and pure PVA nanofibers for 1, 3, 5, 7 days. The viability of HeLa cells on all of drug-loaded nanofibers was significantly lower than pure PVA nanofibers at each time point, indicating that they can inhibit the growth of cancer cells. Moreover, the viability of HeLa cells on Cur-Ms/Dox-nanofibers is the lowest among all samples, which is $<20\%$ on day 7. From the SEM images of HeLa cells cultured on pure PVA nanofibers (left) and Cur-Ms/Dox-nanofibers (right) after 3 days in Figure 5a, we can also find that the cells attached and spread well on pure PVA nanofibers, however, on the Cur-Ms/Dox-nanofibers, cells appear to be spherical shape and shrinkage owing to the cell apoptosis. Therefore, the results indicate that the nanoscale multi-drug delivery system possesses better inhibition effect on cancer cell growth than that of single drug loaded nanofibers.

The inhibition effect of the multi-drug delivery system to HeLa cells in way 2 was shown in Figure 5b. Although cells did not directly contact to the nanofiber meshes, Cur-Ms and Dox can still be released from the nanofibers into the culture medium and have an effect on the cells. During the process, we also investigated the cellular internalization of Cur-Ms and Dox after 4 and 12 h culturing with different samples by CLSM and flow cytometry as shown in Figure S6, Supporting Information. From Figure S6a, Supporting Information, we can find that at 4 h, both the Cur-Ms and Dox released from Cur-Ms/Dox-nanofibers were uptaken by HeLa cells, however, the intensity of the green (Cur) and red (Dox) fluorescence was weaker than that of the free drug groups (Figure S6b,c, Supporting Information), indicating that the two drugs were released slowly. At 12 h, the intensity of both the fluorescences is increased, indicating that more Cur-Ms and Dox were uptaken by cells. According to the release profiles of Cur and Dox in Figure 4b,c, we can conclude that the Cur is intracellular released after the released Cur-Ms are uptaken, and the Dox is directly diffused into the cells due to its good hydrophilicity and rapid release.

From Figure 5b, we can see that the viability of HeLa cells on all of drug-loaded nanofibers are lower than the ones incubated with blank micelles or pure PVA nanofibers, indicating that these drug-loaded nanofibers can still inhibit the growth of HeLa cells in way 2. Comparing with free Cur and all of single drug loaded samples (Cur-Ms, Cur-Ms-nanofibers, Dox-nanofibers), the viability of HeLa cells on Cur-Ms/Dox-nanofibers is still the lowest. The results are matched with the results shown in Figure 5a and demonstrate that the combination of Cur and Dox can enhance the inhibition effect on cancer cells. This is attributed to that Cur can facilitate the retention of Dox in nuclei for a longer period of time and inhibit the resistance of cancer cells to Dox, which promotes the apoptotic response.[41]

However, notably, there is a special case that the cell viability of the free Dox group is the lowest one among all the groups in Figure 5b, which indicates that the cells were more inhibited by free Dox alone and the similar result can be found in Qiu's study.[42] We think that this was due to that the whole free Dox (with the same dosage of dox loaeded in nanofibers) was added into culture medium at the very beginning (the first day), however the Dox loaded in the Cur-Ms/Dox-nanofibers was released into the culture medium during a few time, which resulted in a higher DOX concentration in the medium of the free Dox group than that of the Cur-Ms/Dox-nanofibers group. So HeLa cells cultured with free Dox were killed more and earlier than the one cultured with Cur-Ms/Dox-nanofibers. And since about half of the cells cultured with free Dox were killed and the growth of many cells were inhibited, while cells in the control group were still growing normally, so during the next few days' culture, the cell viability of the free Dox group still got lower and lower.

Figure S7, Supporting Information, shows the fluorescent images of HeLa cells incubated with different samples in way 2. In contrast to the drug fluorescence in the Cur, Cur-Ms, and Dox groups, a continuous fluorescence can be found

in all of the drug-loaded nanofibers groups in 7 days, suggesting that both Dox and Cur-Ms encapsulated in the nanofibers can be released gradually. Moreover, a minimum amount of HeLa cells on Cur-Ms/Dox-nanofibers can be found at 7 days, and the morphology of the cells is spherical, resulted from the cell apoptosis. The result also indicates that the time-programmed release of multiple drugs possesses great potential in improving the efficiency of cancer chemotherapy due to the potential for additive or synergistic effects of multiple drugs. Currently, metastatic cancers, drug resistant cancers, and cancer stem cells impose a great therapeutic challenge,[43,44] and the therapeutic methods available still have many limitations in killing cancer with only one therapeutic agent.[45] The time-programmed release of the multiple drugs from a single formulation is greatly potential for providing an effective resolution for this challenge.

4. Conclusion

In summary, we successfully fabricate a novel multi-agent delivery system loaded with both micelles containing the hydrophobic drug and the hydrophilic drug via a simple electrospinning process. The successful encapsulation of two distinct compounds at separated domains in the nanofibers enables the release of each drug to be independently controlled from a single formulation. Moreover, the problem of the drug leaking in advance from modern micelle systems can be effectively inhibited due to the immobilization of the micelles in nanofiber matrix. Tumor cell inhibition assay reveals that the time-programmed release of multiple drugs possesses great potential in improving the efficiency of cancer chemotherapy and reducing the associated side effect of drugs compared with only one therapeutic agent. Furthermore, the nanoscale multi-agent delivery system as a model system is also potentially suitable for loading other types of drugs, vaccines or growth factors for the combined treatment of diseases such as AIDS and various types of cancers, and the regeneration of damaged organs or tissues based on tissue engineering.

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