

Self-assembled silk fibroin nanoparticles loaded with binary drugs in the treatment of breast carcinoma

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Abstract: Self-assembled nanoparticles of the natural polymer, silk fibroin (SF), are a very promising candidate in drug delivery due to their biocompatible and biodegradable properties. In this study, SF nanoparticles loaded with 5-fluorouracil (5-FU) and curcumin with size 217 ± 0.4 nm and with a loading efficacy of 45% and 15% for 5-FU and curcumin, respectively, were prepared. The in vitro release effect of 5-FU and curcumin from nanoparticles was evaluated as $\sim 100\%$ and $\sim 5\%$, respectively. It has been revealed that the application of such a nanodrug can increase the level of reactive oxygen species, which in turn induces apoptosis of cancer cells in vitro. Animal studies have shown that tumors could be noticeably reduced after being injected with the drug-entrapped nanoparticles. More apoptotic cells were found after 7 days of treatment with SF nanoparticles by a hematoxylin–eosin staining assay. These results demonstrate the future potential of nanoparticle-loaded binary drugs in the treatment of breast cancer.

Keywords: silk fibroin nanoparticles, 5-fluorouracil, curcumin, reactive oxygen species

Introduction

Breast carcinoma is one of the leading causes of death worldwide, and its rate of incidence is increasing every year.¹⁻³ Many strategies have been developed for cancer diagnosis⁴ and therapy, such as immunotherapy, thermal therapy, phototherapy, surgery, gene therapy, chemotherapy, and radiotherapy.⁵⁻¹¹ Each of these methods has its own advantages and disadvantages.¹² Although small molecules have been used for chemotherapy for many years, there are several disadvantages, including poor water solubility, nonspecific targeting and misdistribution, and drug resistance shortly after initial treatment. These disadvantages hinder their efficiency and effect in treating cancers. Recently, delivery of these small molecules by using nanoparticles has been rapidly developed, which offers a new chance for cancer treatment in the preclinical or clinical phases.^{13,14} The unique properties of nanoparticles could overcome the limitations of using small molecules as therapeutic agents in biomedical applications.¹⁵ For example, the uptake of drug could be enhanced by the “enhanced permeability and retention effect”,¹⁶ which can increase the drug accumulation in target cancer tissues and reduce the efflux pump-mediated drug resistance. Besides, encapsulated by nanoparticles, the solubility and half-life of small molecular drugs can be improved with controllable releasing behaviors.

Curcumin is a natural compound with diphenolic groups derived from turmeric *Curcuma longa*, which is widely used in chemotherapy/radiosensitizing of cancer¹⁷⁻¹⁹ and other biomedical fields^{20,21} with almost no toxicity,^{22,23} which has potential as a promising candidate in clinical trials for cancer treatment. However, owing to its poor

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solubility in physiological environment, curcumin often shows a low level of absorption inside the gut, and the amount of curcumin that reaches the tissues outside the gut is pharmacologically insignificant as per the measurements of blood plasma levels and biliary excretion.²⁴ 5-Fluorouracil (5-FU) is another highly efficient chemotherapeutic agent that inhibits the methylation reaction of deoxyuridylic acid to thymidylic acid.^{25,26} This chemical drug can cause intense thymine deficit in the cells by interfering with the synthesis of deoxyribonucleic acid and ribonucleic acid, which eventually results in an imbalanced growth and death of cancer cells. To increase their water solubility and biocompatibility, several drug carriers are developed based on nanotechnology,²⁷ ranging from liposome,²⁸ proteins,²⁹ saccharides,³⁰ phospholipids,³¹ to inorganic silica particles^{32,33} and polymeric organic nanoparticles.^{34–36} Besides, the slow and controllable release of drugs is helpful in providing a sustainable dose at a therapeutic level, which benefits the improved efficiency and safety. Recently, curcumin when encapsulated with silk fibroin (SF) nanoparticles and administered to breast cancer cells was observed to have a greatest entrapment, intracellular uptake, and controlled release.²⁹ Moreover, it has also been revealed that administration of two or more drugs concomitantly is also important in cancer treatment.

Owing to its biocompatible and biodegradable properties, natural polymer SF is considered to be a very promising candidate in biomedical fields.^{37–42} As a drug carrier, the SF molecules possess controllability of their degradation under *in vitro* and *in vivo*, which can be regulated by changing its size, crystallinity, molecular weight, and degree of cross-linking.^{43,44} The hydrophobic amino acid residues in SF molecules, such as alanine, glycine, and tyrosine, can enhance the entrapping of curcumin and 5-FU through hydrophobic interaction and π - π packing, which increase the loading efficacy of the binary drug carrier. Besides, the hydrophilic amino acid residues in SF molecules, such as serine, aspartic acid, and glutamate, increase their water solubility and induce the formation of nanoparticles in aqueous solutions. These properties make SF molecules a good candidate as drug carrier.⁴⁵ In this study, we developed SF nanoparticles as a degradable and biocompatible carrier for loading and delivering binary drugs curcumin and 5-FU, which showed an excellent anticancer performance *in vitro* and *in vivo*.

Materials and methods

Preparation of 5-FU and curcumin loaded with nano-SF particles

SF aqueous solution was prepared as previously described.⁴⁶ Bombyx mori domestic silk was treated with Na_2CO_3

solution to remove sericin. Briefly, 80 g of raw silk fibers was degummed three times in 4,000 mL of 0.075% sodium carbonate and then rinsed thoroughly with deionized water. After drying in an oven at 60°C, the extracted SF was dissolved in 9.3 M LiBr solution at 60°C for 4 hours. About 8.0% (w/v) SF solution was obtained after 4 days of dialysis in deionized water, which was followed by filtration. Curcumin and 5-FU were obtained from Aladdin Industrial Corporation of China and Sangon Biotechnological Group (Shanghai, People's Republic of China), respectively.

Curcumin (1 mg/mL) and 5-FU (1 mg/mL) were dissolved in alcohol; 5-FU solution was added into 1% SF solution in drops and then curcumin solution was also added into the mixed solution in drops. 5-FU and curcumin nanoparticles that were loaded with SF were prepared by stirring for 1 day; then they were centrifugated at 12,000 rpm for 30 minutes and dialyzed for 24 hours.

Morphology and structure of nanoparticles

Nanoparticles were observed through a transmission electron microscope (Tecnai™ G2 Spirit BioTwin; FEI Company, Hillsboro, OR, USA) at a magnification of 120 kV. Briefly, a drop of aqueous solution of lyophilized solutions (5 mg/mL) was placed on a bronze ring membrane. The samples were air dried at room temperature before loading onto the microscope.

Fourier transform infrared spectroscopy was performed with Scimitar 1000 (VARIAN Medical Systems, Inc, Palo Alto, CA, USA). The wave number ranged from 200 to 4,000 cm^{-1} and the distinguished ability was 2.0 cm^{-1} .

Dynamic light scattering measurements

Dynamic light scattering measurements for determining the average size and size distribution of the polymeric micelles were performed using a Nanosizer 90ZS (Malvern Instruments, Malvern, UK). The solution was filtered with membrane extruder having an average pore size of 0.2 μm (Lipex Biomembranes Inc., Vancouver, Canada). All the data analyses were performed in automatic mode. Measured size was presented as the average value of 20 runs, with triplicate measurements within each run.

Drug loading content of nanoparticles, encapsulation efficiency, and release effect

To determine the encapsulation efficiency (EE), 2 mL of nanoparticles was dispersed in LiBr and the absorbance was measured at 265 and 425 nm. For comparison purposes, UV-vis (ultraviolet-visible) spectra of SF solution, free curcumin, and free 5-FU were also used.

To determine the release effect, 2 mL of nanoparticles was added to 18 mL of phosphate-buffered saline (PBS) and shaken at a rate of 120 rpm at 37°C. The medium (2 mL) released was collected for measurement at a specified time and 2 mL of fresh buffer was added again. A dialysis bag with a molecular weight of 3,500 was cut for dialyzing the free drugs to the outside medium.

The controllable releasing capability was determined by standard curve method. The releasing percentage of the two drugs is defined as the concentration of the ratio of dialyzed solution to the saturated loading concentration inside the nanoparticles. The amounts of 5-FU and curcumin released from nanoparticles were determined by measuring their absorbance at 265 and 425 nm, respectively.

The drug *EE* was calculated by the following equation: $EE = m_{d,n}/m_{d,f} \times 100\%$, where $m_{d,n}$ and $m_{d,f}$ are the weight of the drug in nanoparticles and the feeding drugs, respectively. According to a previous report,⁴⁷ the cumulative content of the drugs released can be calculated by the following equation:

$$R\% = V_e \sum_{i=1}^{n-1} C_i + V_0 C_n, \quad (1)$$

where V_e is the volume of the released solution collected at each time point (2 mL), V_0 is the volume of original buffer solutions (18 mL), C_i is the drug concentration in the released medium at displacement time i , and n is the total number of displacement. C_n is the final concentration in the released medium.

4T1 culture

The 4T1 cells (murine breast cancer cells) were cultured in standard cell media as recommended by the American Type Culture Collection (ATCC) in RPMI-1640 medium containing 10% (v/v) fetal bovine serum and 1% penicillin–streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in 5% CO₂ humidified incubator.

Cell apoptosis analysis by flow cytometry

4T1 cells were cultured at 2×10^5 cells for 1 day, and then fibroin loaded-curcumin and 5-FU nanoparticles, control and free curcumin and 5-FU at 37°C for 48 h respectively were added. The cells were harvested by trypsinization and washed twice with PBS. The cells were centrifugated at 800 rpm for 5 minutes and added to PE Annexin V Apoptosis Detection Kit I (BD Bioscience, San Jose, CA, USA) at room temperature for 1 hour. The cell suspension was then analyzed with FACSVerse™ flow (BD Bioscience).

Reactive oxygen species

The reactive oxygen species (ROS) contents were measured by flow cytometry. Briefly, 4T1 cells (2×10^5 /well) were cultured on dishes and allowed to grow overnight, and then curcumin and 5-FU loaded with nano-fibroin and free curcumin and 5-FU were added at specified concentrations to dishes. The cells were digested with trypsinization after 24 hours of drug-loaded nanoparticles treatment and then stained by 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Beyotime Biotech, Nantong, People's Republic of China) at 37°C for 30 minutes. Flow cytometry was used for detecting the fluorescence of the oxidizing by-product, dichlorodihydrofluorescein (DCF) under the laser of 488 nm UV light. The fluorescence was analyzed using FACSVerse™ flow (BD Bioscience).

In vivo experiment

4T1 cells were harvested at 1×10^6 cells and 100 µL was injected into the abdomens of SPF BALB/C mice aged 5–8 weeks. Thirty mice bearing 4T1 tumor tissues for 1 week (7.5 mm) were divided into control, free group, and nano group. A total of 100 µL of nanoparticle-loaded drug, free curcumin, and 5-FU, whose concentration was 450 µg/mL, were percutaneously intra-tumor injected. The size of the tumor tissues was observed after 3, 5, and 7 days of treatment. Some tissues were prepared for hematoxylin–eosin staining.

All animal experiments were conducted in accordance with Management Ordinance of Experimental Animal of China ([2001] No 545) and approved by the Jiangsu Province in experimental animals management rules ([2008] No 26).

Results

Figure 1 shows the physical characterization of the SF nanoparticles loaded with 5-FU and curcumin. The typical transmission electronic microscopic image (Figure 1A) revealed that the SF molecules conjugate with each other to form nanoparticles with a size of 50–250 nm (Figure 1B). The hydrodynamic diameter (D_h) obtained by dynamic light scattering shows that the average D_h is 217 ± 0.4 nm, with a polydistribution index of 0.049–0.059, and thereby nanoparticles disperse well in aqueous environment.

The characterizations of SF nanoparticles studied by the Fourier transform infrared spectroscopy are shown in Figure 1C. The results show that SF nanoparticles did not undergo any change compared to pure SF, which emphasizes that the chemical bonding remained unchanged after the SF nanoparticles were loaded with the drugs. The characteristic peak of 5-FU group was found in the following regions of SF nanoparticles, 3,000–2,900 cm⁻¹ (–C–H stretching),

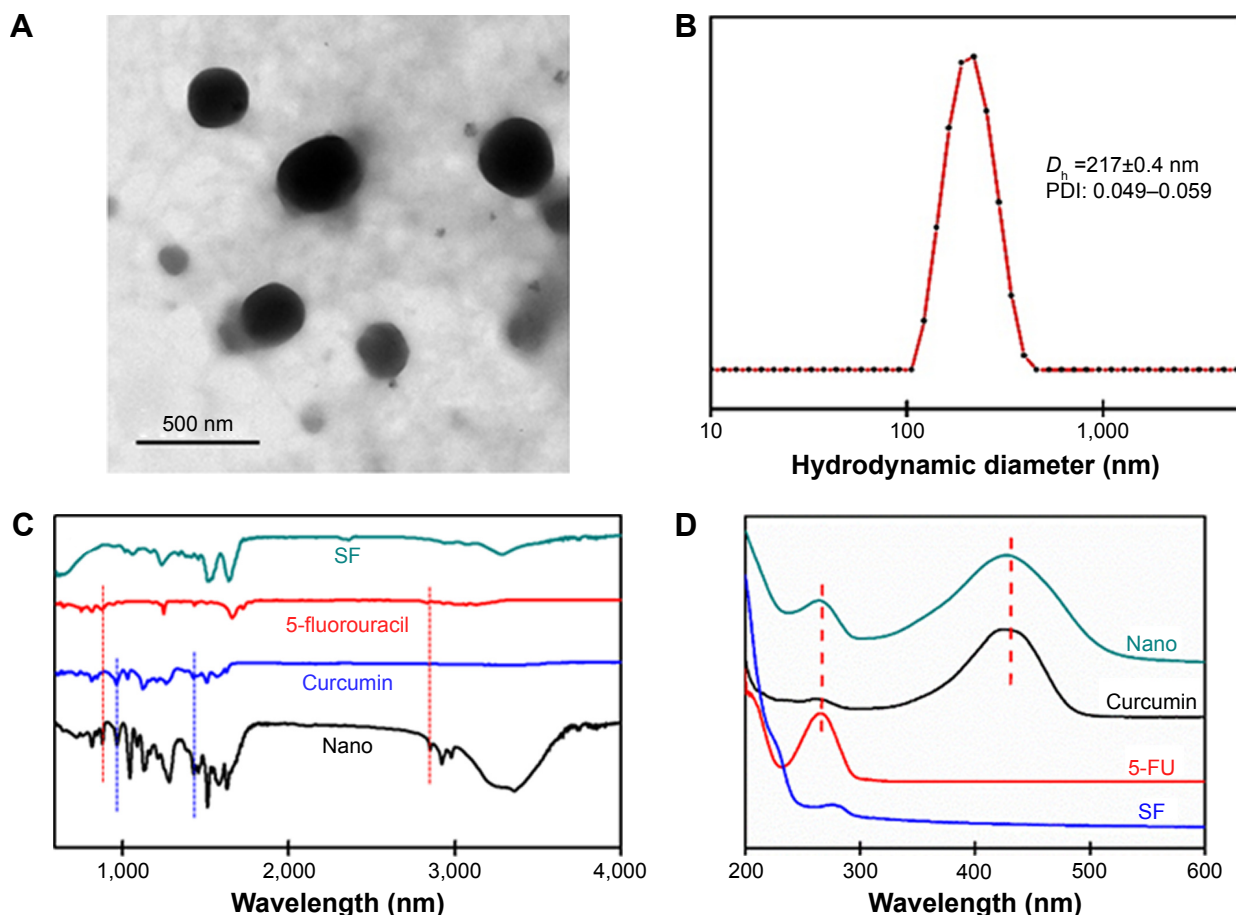


Figure 1 Characterization of silk fibroin (SF) nanoparticles after loading with 5-fluorouracil (5-FU) and curcumin.

Notes: (A) TEM and (B) hydrodynamic diameter obtained by DLS. (C) FTIR. (D) UV-vis curve of the binary drug-loaded nanoparticles (Nano), free curcumin, free 5-FU, and SF, respectively.

Abbreviations: TEM, transmission electron microscopy; FTIR, Fourier transform infrared spectroscopy; UV-vis, ultraviolet visible spectroscopy; DLS, dynamic light scattering.

$1,425\text{ cm}^{-1}$ corresponding to the $\text{C}=\text{N}$ and $\text{C}=\text{C}$ ring stretching vibrations, and pyrimidine band at $1,246\text{ cm}^{-1}$. Curcumin showed its characteristic group absorption peaks in sharp absorption bands at $1,605\text{ cm}^{-1}$, $1,502\text{ cm}^{-1}$ ($\text{C}=\text{O}$ and $\text{C}-\text{C}$ vibrations), $1,435\text{ cm}^{-1}$ (olefinic $\text{C}-\text{H}$ bending vibration), and $1,285\text{ cm}^{-1}$ (aromatic $\text{C}-\text{O}$ stretching vibration).

The UV-vis spectrum also reveals that 5-FU (265 nm) and curcumin (425 nm) were successfully loaded into the fibroin nanoparticles (Figure 1D). The drug contents of the nanoparticles were measured and compared with the feeding drug content by UV-vis spectrum, which yielded the value of drug EE. The result shows that the drug was successfully loaded into the nanoparticles by self-assembly, with an EE value of 45% and 15% for 5-FU and curcumin, respectively. As shown in Figure 2, the release rate of SF nanoparticles was up to ~100% and ~5% for 5-FU and curcumin, respectively.

To verify the efficacy of drug-loaded nanoparticles in treating cancer, their effectiveness was first evaluated at cellular level, by examining the cellular morphological changes

and sensitivity toward cell apoptosis. The morphology of the cells was found to be normal; cells of the control group grew well for 2 days (Figure 3A), whereas the cells incubated with free 5-FU and curcumin group suffered slight

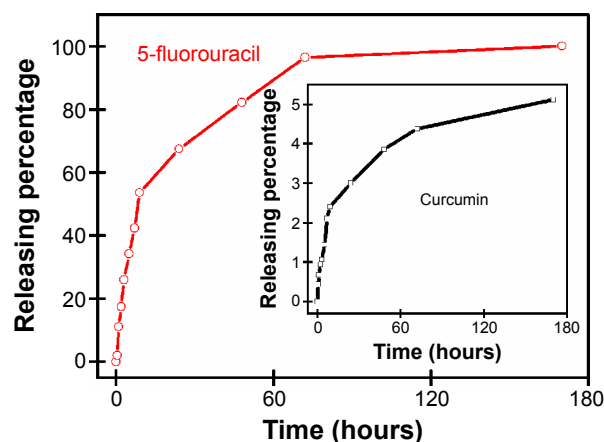


Figure 2 Releasing percentage of 5-fluorouracil and curcumin from silk fibroin nanoparticles.

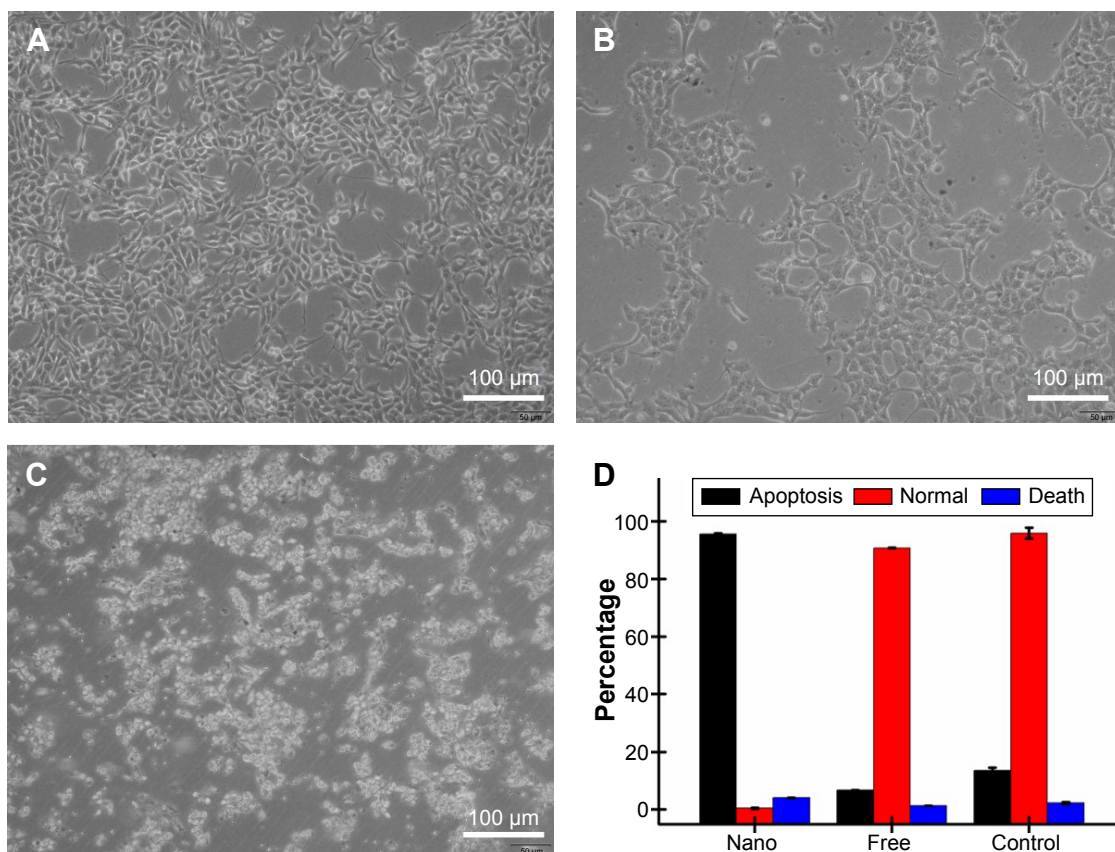


Figure 3 Morphologies of 4T1 cells after incubation with (A) control, (B) free 5-fluorouracil and curcumin, and (C) drug-loaded nanoparticles, respectively. (D) Flow cytometry results that show the apoptosis of 4T1 cell lines at same incubating conditions, (Magnification 100×).

apoptosis (Figure 3B). The cells incubated with drug-loaded nanoparticles suffered serious apoptosis after 2 days of culture, and their shape changed from spindle form to spherical form (Figure 3C). These results indicate that the drug-loaded nanoparticles exhibited chemotherapeutic effect toward the 4T1 cells. These results were further confirmed by apoptosis assay. As shown in Figure 3D, the statistical percentage of cell apoptosis of drug-loaded nanoparticles was more than 90%, which was much more than that for free drugs (~8%) and control (~2%).

The ROS level was determined by the fluorescence intensity of DCFH-DA. As shown in Figure 4, the fluorescence intensity was found to be 33 ± 2 and 22 ± 1 for the drug-free nanoparticles and free drugs, respectively. The ROS level almost showed no change after incubating the cells with free drugs. In contrast, the ROS level became very high (323 ± 12) after incubating the cells with drug-loaded nanoparticles. ROS level in silk fibroin loading curcumin and 5-FU nanoparticles can be enhanced up to tenfold more than that of control. Other results show that drug-loaded nanoparticles group showed more apoptosis (Figure 3), which was probably due to the generation of ROS inside the cells.

The efficacy of the drug-loaded nanoparticles in the treatment of cancer was further investigated *in vivo*. The binary drug-loaded nanoparticles were injected into tumor-loaded mice and the size of tumor was then measured. As shown in Figure 5A, the binary drug-loaded nanoparticles obviously reduced the size of tumors. In contrast, the tumor size

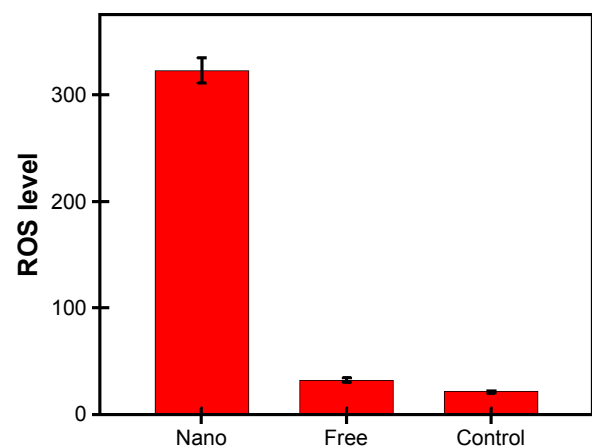


Figure 4 Increase in the intracellular level of ROS observed after the 4T1 cell lines were incubated with drug-loaded nanoparticles compared to free 5-fluorouracil and curcumin, respectively.

Abbreviation: ROS, reactive oxygen species.

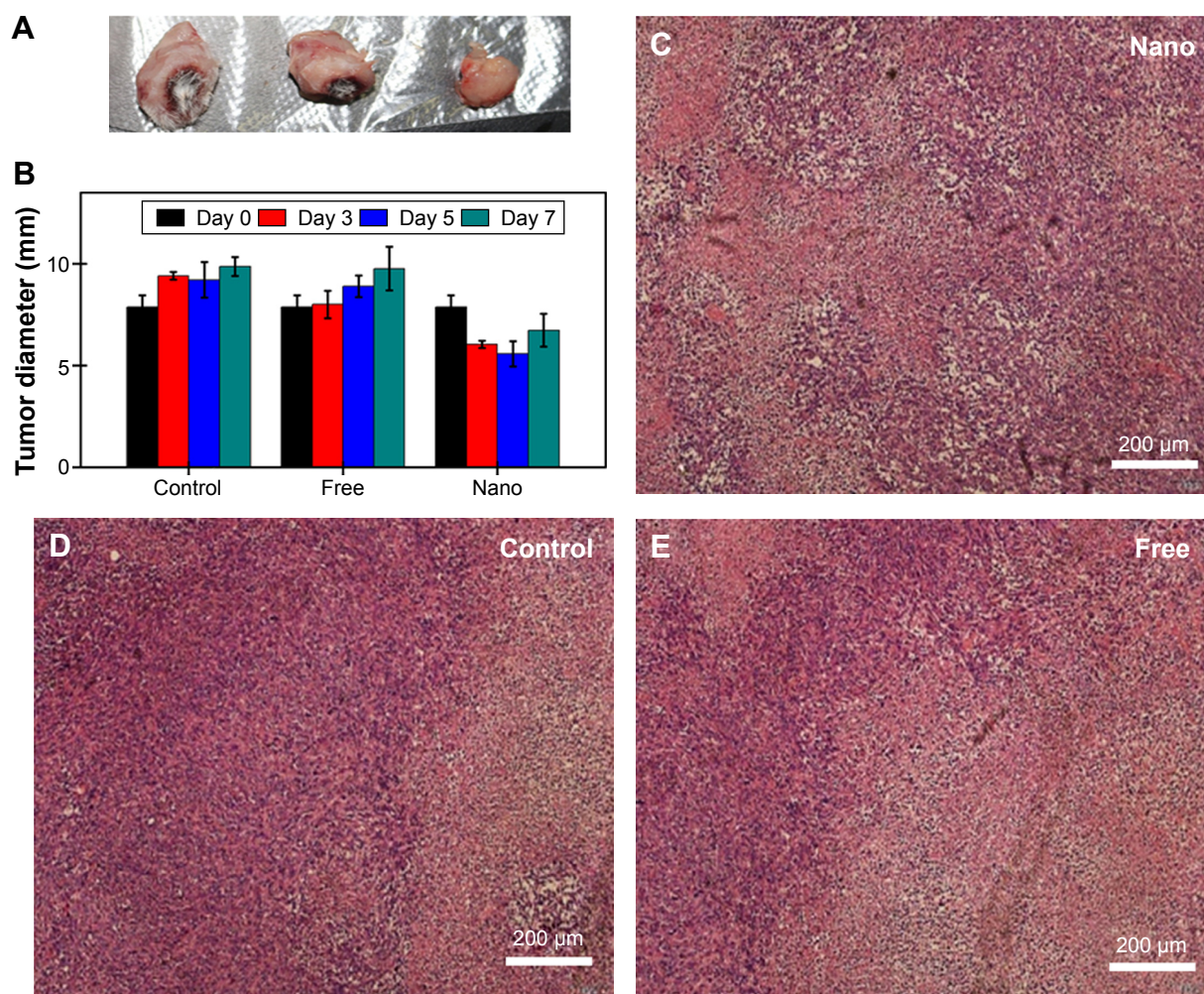


Figure 5 Changes after treatment with drugs in size and histochemistry.

Notes: (A) Optical image that shows the tumor reduction in mice injected with nanoparticle-loaded drugs. (B) Statistic analysis of tumor diameter observed in groups administered with free binary drug, nanoparticle-loaded binary drug, and control (PBS buffer). (C–E) HE staining of tumor tissues. Magnification 100 \times .

Abbreviations: PBS, phosphate-buffered saline; HE, hematoxylin–eosin.

was reduced to a little extent when treated with free drug, and almost no changes can be observed compared with the control group. Measurement of tumor size from the three experimental groups, as shown in Figure 5B, showed that injection of drug-entrapped nanoparticles reduced the tumor size on the third and fifth days, but slightly increased on the seventh day. In contrast, the treatment of free drug only shows the reduced increment of tumor and the increment again accelerates for 5–7 days. These comparisons indicate that entrapment of 5-FU and curcumin into SF nanoparticles exhibited efficacy in *in vivo* treatment of breast cancer. No injection was given after 7 days – the mice in the control group died after 25 days of injection, in free-drug group after 33 days, and in nano-group after 45 days. To assess whether SF nanoparticles induced tissues apoptosis, we evaluated the tumor sections histologically by H&E. More apoptotic cells were found in the group treated with SF nanoparticles

(Figure 5C) after 7 days of treatment compared to those in the free group or control group (Figure 5D and E).

Discussion

The drug 5-FU is a chemotherapeutic agent highly effective in the treatment of cancer, which inhibits the methylation reaction of deoxyuridylic acid to thymidylic acid, the key repair process for reducing thymine deficits.^{25,26} Curcumin is widely used as an anticancer agent in preclinical and clinical trials, probably due to their capability of generating intracellular ROS. However, this drug is poorly soluble in water, which restricts it from being directly used in aqueous solutions. On being carried by SF nanoparticles with well-dispersion capacity, more drugs can exist inside aqueous solutions, which can then increase the effectiveness of the drugs in treating cancer cells and *in vivo* tumor cells via incubation or injection. In addition, curcumin enhances

the solubility of 5-FU inside the nanoparticles through π - π packing. Although the cumulative release of curcumin is less, all curcumin will be released from SF nanoparticles in a stepwise process as a result of degradation.

In comparison with the free drugs, the nanoparticle-loaded drug can help a larger amount of drugs enter the cells or tumor tissue with controllable releasing capability. This possibly induces the apoptosis of cancer cells (or decreases the cell variability). A previous report⁴⁸ also has found a similar result. Apoptosis can be induced by the ROS generated after the administration of curcumin.^{49–52} ROS could act as a vital candidate pathway for inducing tumor cell apoptosis by targeting mitochondrial and endoplasmic reticulum stress-related death pathway.⁹ As a result, the releasing effect of these two drugs is comparable based on their mass percentage, which may help them in treating cancer cells. Our experimental results revealed that 5-FU and curcumin loaded with SF nanoparticles have an increased anticancer efficacy compared to free 5-FU and curcumin.

Conclusion

In conclusion, we used SF nanoparticles to entrap the binary drugs 5-FU and curcumin to investigate their effectiveness on breast cancer in vivo and in vitro. The results showed significant improvement in the cytotoxic activity and bioavailability of SF nanoparticles compared to free curcumin and 5-FU. The anticancer effect observed may be induced by the apoptosis of cells via the generation of cellular ROS. Self-assembling polymer systems of SF-loaded 5-FU and curcumin will be a facile method for cancer treatment.

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Author contributions

All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflict of interest in this work.

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