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pH-Dependent anticancer drug release from silk nanoparticles

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Abstract

Silk has traditionally been used as a suture material because of its excellent mechanical properties and biocompatibility. These properties have led to the development of different silk-based material formats for tissue engineering and regenerative medicine. Although there have been a small number of studies about the use of silk particles for drug delivery, none of these studies have assessed the potential of silk to act as a stimulus-responsive anticancer nanomedicine. This report demonstrates that an acetone precipitation of silk allowed the formation of uniform silk nanoparticles (98 nm diameter, polydispersity index 0.109), with an overall negative surface charge (-33.6 ± 5.8 mV), in a single step. Silk nanoparticles were readily loaded with doxorubicin (40 ng doxorubicin/ μ g silk) and showed pH-dependent release ($\text{pH } 4.5 \gg 6.0 > 7.4$). *In vitro* studies with human breast cancer cell lines demonstrated that the silk nanoparticles were not cytotoxic ($\text{IC}_{50} > 120 \mu\text{g/ml}$) and that doxorubicin-loaded silk nanoparticles were able to overcome drug resistance mechanisms. Live cell fluorescence microscopy studies showed endocytic uptake and lysosomal accumulation of silk nanoparticles. In summary, the pH-dependent drug release and lysosomal accumulation of silk nanoparticles demonstrated the ability of drug-loaded silk nanoparticles to serve as a lysosomotropic anticancer nanomedicine.

Keywords

adriamycin; *Bombyx mori*; nanomedicine; fibroin

1. Introduction

Research over the past three decades has facilitated studies such that more than 40 nanomedicines that are now in routine clinical use.¹ Nanomedicines are defined as specifically engineered, nanosized drugs and drug delivery systems that are composed of multiple components.¹ Spurred by the success of nanoparticulate imaging agents (SPIONS family)¹ there has been a revival in nanoparticle research. More than a dozen nanoparticles are in early clinical development for a wide range of indications, including cancer, diabetes, hypercholesterolemia, seasonal influenza, and smoking cessation.²

The payloads of these nanomedicines differ widely. However, when targeting cancer, there is a universal requirement for nanoscale therapeutics to reach the tumor microenvironment and often to deliver the payload to a specific intracellular compartment for the desired therapeutic effect.³ Because nanosized drug delivery systems only gain access into cells through the process of endocytosis⁴ these carriers often end up in lysosomes where they are

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exposed to a low pH (typically pH 4.5) and proteolytic enzymes. This lysosomal microenvironment can be exploited to trigger drug release from the carrier (lysosomotropic delivery)⁵ using stimulus-responsive polymers.³ These synthetic polymers raise concerns for toxicity¹ or the impact of residuals in cells due to their limited biodegradability. In particular, lysosomal accumulation leading to lysosomal storage diseases and/or inadequate renal excretion are potential caveats with the use of such synthetic polymers in these systems.⁶ Therefore, second-generation polymers have been engineered to contain proteolytically or hydrolytically cleavable sites in an attempt to overcome these shortcomings.⁷ Due to these limitations, biopolymers such as chitosan, alginate, dextran/dextrin, and cyclodextrin have been explored as alternatives to synthetic systems.⁸ While many of these systems are regarded as biocompatible, there is a need to chemically modify these polymers to yield stimulus-responsive nanomedicines. For example, chitosan is frequently quaternized to maintain aqueous solubility and to facilitate nanoparticle formation.⁹ Additionally, mixtures of chitosan with acrylic acid monomers are *in situ*-polymerized to generate hollow nanoparticles.¹⁰ Similar strategies have been used for other biopolymers, such as alginate, that are functionalized with poly[2-(diethylamino)ethyl methacrylate] to form self-assembling nanoparticles.¹¹ Although these native biopolymers are regarded as biocompatible, their chemical modification leads to products that have no track record in terms of acute or chronic impact on cells and tissues.

Silk proteins offer unique material properties, proven biocompatibility and versatility to generate various material formats such as films, gels, fibres, mats or composites under mild processing conditions, without chemical modifications, which has allowed silk to expand beyond its traditional use as suture material.¹² There have been a number of studies focused on generating silk particles using the following methods: (1) polyvinyl alcohol blends (particle size range from 300 nm to 10 μ m)¹³, (2) vibrational splitting of a laminar jet (particle size range 100 to 400 μ m)¹⁴, (3) spray-drying (5- μ m particles)¹⁵, (4) salting out (particle size range 1 to 2 μ m)¹⁶, (5) capillary microdot printing (particle size range 25 to 140 nm)¹⁷, or (6) organic solvents (particle size range 35 to 170 nm).^{18, 19} The majority of these studies have focused on the processing parameters to generate the particles, while a small number of studies have examined the ability of silk particles to entrap and release (model) drugs.^{13, 16, 18} The majority of these studies have used silk derived from silk cocoons (*Bombyx mori*)¹³⁻¹⁹; notable exceptions have been studies that have used recombinantly expressed spider silks to generate microcapsules²⁰ or nanoparticles using chimeric constructs such as silk-elastin-like proteins²¹ or lysine modified spider silks²².

However, the potential of silk to act as a stimulus responsive drug delivery system in the context of anticancer nanomedicine has not been addressed and was the goal of this present study. To address this goal, a robust and simple method was used to generate nanoparticles with a narrow size distribution. The particles formed in the process were evaluated as a putative anticancer drug delivery vector. The particles were examined for stimulus responsiveness and the intracellular trafficking was studied in human breast cancer cells.

2. Results

2.1 Silk nanoparticle generation, characterization and loading with doxorubicin

A one-step desolvation procedure was applied to generate silk nanoparticles ($23 \pm 8\%$ efficiency) of uniform size (98 nm) that showed a low polydispersity (0.109) and an overall moderate negative net charge of -33.6 ± 5.8 mV (Figure 1). Qualitative characterization by SEM indicated that the silk nanoparticles had a spherical geometry (Figure 1 b).

Next, we examined the ability of these silk nanoparticles to serve as a potential drug delivery system (Figure 2). In some cases silk is known to facilitate the adsorption of weakly

basic drugs²³, therefore we tested the binding and release characteristics of silk nanoparticles for doxorubicin. Using either 500 μg or 125 μg of the silk nanoparticles, the binding characteristics of doxorubicin was studied (Figure 2a); at concentrations of up to 0.04 μg doxorubicin/ μg of silk, >95% of the drug could be absorbed by the silk. Going beyond this ratio by further increasing the amount of doxorubicin in the system, the percentage of bound drug declined while the total amount of drug loaded per silk weight remained constant at approximately 40 ng doxorubicin/ μg silk. Next, we examined the release characteristics of doxorubicin from the doxorubicin-loaded silk nanoparticles at different pHs (Figure 2b,c). Doxorubicin-associated fluorescence was monitored in the buffers with pH values mimicking those of blood plasma (pH 7.4), early endosomes (pH 6.0), and lysosomes (pH 4.5). Throughout the early time points (1 to 24 h) and the 7-day release period, drug release was significantly faster (2 to 3 fold) from the doxorubicin-loaded silk nanoparticles that were incubated at pH 4.5 than for the other test conditions.

2.2 Cellular response toward silk nanoparticles

First, we examined the cytotoxicity of the silk nanoparticles using human breast cancer cell lines. Both cell lines under normoxic and hypoxic conditions demonstrated >90% viability with no significant difference compared to control cultures at the maximum tested silk nanoparticle concentration (IC_{50} > 120 $\mu\text{g}/\text{ml}$) (Figure 3a). Next, we examined the long-term response of human breast cancer cell lines to either the doxorubicin-loaded silk nanoparticles or free drug using an equivalent doxorubicin dose (Figure 3b). MCF-7/^{WT} cells demonstrated a comparable response (6% viability) to free doxorubicin and silk nanoparticle-delivered doxorubicin under normoxic and hypoxic conditions (Figure 3b). For MCF-7/^{DOX} cells, significantly higher doses of doxorubicin were needed to achieve a therapeutic response (IC_{50} 4 $\mu\text{g}/\text{ml}$ versus 0.1 $\mu\text{g}/\text{ml}$ for MCF-7/^{DOX} and MCF-7/^{WT} cells, respectively) (Supplementary Figure 1). However, when the cells were exposed to the doxorubicin-loaded silk nanoparticles, the cytotoxicity was significantly improved (76% viability) compared to the equivalent amount of free drug (91% viability) (Figure 3b). This response was observed for both normoxic and hypoxic culture conditions.

2.3 Cellular uptake and trafficking of silk nanoparticles

With the aid of live cell confocal microscopy, the intracellular fate of the silk nanoparticles was examined. Both MCF-7/^{WT} and MCF-7/^{DOX} cells demonstrated typical endocytic labeling of vesicular structures (Figure 4). To discriminate between different vesicular types, lysosomes were labeled with LysoTracker Red to highlight these acidic intracellular organelles. Single confocal sections demonstrated that the silk nanoparticles co-localized with the lysosomes (Figure 4a, b).

3. Discussion

Silk has been used for centuries in humans as a suture material due to its strength, handling, durability, and biocompatibility.¹² Recently silk has also been approved by the FDA for new medical applications, such as surgical meshes (Allergan Medical, Inc.). The biocompatibility of silk and its remarkable material properties have allowed the development of a diverse spectrum of material formats that can be utilized for a broad range of biomedical applications. For example, we have recently described the first examples of self-assembling silk hydrogels for anticancer drug delivery²⁴ and silk films for focal therapy of breast cancer.²³ To go beyond localized (chemotherapy) drug delivery, we set out to examine the potential of silk to serve as a stimulus-responsive nanomedicine.

First, we tested a number of solvents to generate silk nanoparticles (data not shown) and also found acetone to be useful to yield uniform nanoparticles as detailed elsewhere.¹⁹ Exposing

silk to organic solvents, such as methanol, ethanol or acetone, dehydrates silk fibroin, facilitating closer chain packing of the hydrophobic Gly-X repeats that are responsible for β -sheet formation (crystalline region) and particle stabilization through these physical crosslinks.²⁵ Second, we examined the impact of extraction time (5, 20 and 60 min) during fibroin preparation from silk cocoons on nanoparticle formation and found that silk extracted for 60 min (boiling time) provided the best results in terms of particle preparation and handling. During this extraction, the amorphous regions of silk are progressively degraded, resulting in shorter silk fragments while sparing the crystalline regions of the protein.²⁶ The overall reduced molecular weight of silk likely facilitates nanoparticle formation, which driven in part by more favorable thermodynamics. However, an extraction time of 5 or 20 min during fibroin preparation induced substantial gelation of the silk once it was added to the acetone. The subsequent low particle recovery precluded the set of experiments conducted with the 60 min extracted silk. It is therefore unknown how boiling time impacts particle characteristics, drug binding and release. Furthermore, we have not attempted to fractionate silk (nano)particles. The above-mentioned caveats are currently being addressed in a separate study.

We first examined the impact of low pH on the ability of silk nanoparticles to deliver a therapeutic payload because nanomedicines often accumulate in lysosomes. Previous electrokinetic measurements on silk model substrates indicated how environmental pH impacts the charge characteristics of silk; at low pH values silk loses its overall acidic surface properties and negative net charge.²⁷ To exploit these characteristics, we selected doxorubicin because it is a weak base and adsorbs to silk (in part) by electrostatic interactions. Silk has a high doxorubicin-binding capacity at pH 7.4, providing a loading efficiency of 40 ng doxorubicin/ μ g silk, values that correlate well with those established for silk films.²³ Studies examining silk/drug interactions by our group²³ and others²⁸ have demonstrated that the $\log D$ of weak bases is a critical parameter that governs drug adsorption to silk. Therefore, the low pH values encountered in lysosomes were predicted to increase drug release by changing the electrostatic interaction between the silk and doxorubicin (Figure 5). Next, the lysosomotropic drug delivery potential of silk was simulated using a range of pHs that reflects the pHs of blood plasma (pH 7.4), early endosomes (pH 6.0), and lysosomes (pH 4.5). The release rates for the pH 4.5 samples were significantly higher than for the other conditions, indicating that silk nanoparticles could serve as a lysosomotropic drug delivery platform. A large number of synthetic and biopolymer-based delivery systems have been engineered to serve as a lysosomotropic delivery systems. However, there is a lack of natural biopolymers with the inherent ability to respond to changes in pH to trigger drug release. This is an important outcome of the present study, that a native biopolymer without any chemical modifications is able to elicit drug release in response to pH.

Targeted drug delivery and overcoming multidrug resistance are major driving forces for the development of nanomedicines. For example, packaging small molecular weight drugs into nanoparticles changes the cellular and whole body pharmacokinetics of the drugs.²⁹ In solid tumors, the leaky blood vessels and reduced lymphatic drainage of the tumor vasculature facilitate the passive accumulation of nanomedicines in the tumor tissue. This phenomenon is called the enhanced permeation and retention effect (EPR).³⁰ The EPR effect is often a key factor that permits (passive) targeting of nanomedicines to solid tumors. Once in the extracellular space of tumors, nanomedicines are endocytosed by cells and accumulate in intracellular organelles, such as lysosomes.⁴ The endocytic uptake of nanomedicines provides an opportunity to overcome drug resistance mechanisms, which effectively efflux small molecular weight drugs that normally access the cytoplasm by diffusing across the plasma membrane.²⁹ To test the ability of our doxorubicin-loaded silk nanoparticles to serve as a lysosomotropic drug delivery platforms that can overcome drug resistance, we have

used human breast cancer cells that are resistant to doxorubicin (MCF-7/^{DOX}).³¹ The drug-loaded silk nanoparticles were significantly more cytotoxic than the free drug at the doxorubicin equivalent dose. In contrast, the parental cell lines (MCF-7/^{WT}) showed an excellent response to doxorubicin regardless of the mechanism of drug delivery. This result suggested that doxorubicin-loaded silk nanoparticles were able to overcome the resistance mechanisms of MCF-7/^{DOX} cells by changing the cellular uptake mechanism. To recapitulate the tumor microenvironment, cytotoxicity studies were also conducted under hypoxic conditions. While intracellular pH is known to affect doxorubicin distribution³² and is predicted to have an impact on the ability of silk nanomedicines to act as lysosomotropic drug delivery systems, the effect of oxygen tension has not been studied in this context. The data presented in this study indicated that hypoxic conditions did not influence the biological response in MCF-7/^{WT} and MCF-7/^{DOX} toward doxorubicin. Using live cell confocal microscopy, we have been able to demonstrate that silk nanoparticles accumulated in lysosomes, further supporting the notion of lysosomotropic drug delivery. Overall, it is encouraging that the drug-loaded silk nanoparticles worked more efficiently than free doxorubicin because for many nanomedicines, the therapeutic potential is only observed in animal models.³³

This study also examined the cytotoxicity of silk nanoparticles alone. Over the tested concentration range, no cytotoxicity was observed. While silk is generally accepted as biocompatible, changing the scale of the material can have a major biological impact. For example, carbon nanotubes induced cell death due to “frustrated” endocytosis, whereas the same material at the micron scale was not cytotoxic.⁴

4. Conclusion

Silk nanoparticles of defined surface charge and size were generated that were readily loaded with a chemotherapeutic agent and used as a nanomedicine. These silk-based nanoparticles were able to serve as a lysosomotropic delivery platform and overcame drug resistance *in vitro*. These results establish a foundation for the development of more advanced silk-based nanomedicines.

5. Experimental Section

In vitro cytotoxicity studies: The wild-type (WT) human breast cancer cell line MCF-7 (denoted as MCF-7/^{WT}) was obtained from ATCC (Manassas, VA, USA), and the doxorubicin-resistant cell line MCF-7/^{DOX} was a gift from Dr. Kapil Mehta (MD Anderson Cancer Center, University of Texas, USA).³¹ The cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C, and cultures were routinely subcultured every 2 to 3 days unless specified otherwise. The MCF-7/^{DOX} cells were grown in RPMI 1640 with 10% v/v FBS and 2 µg/ml doxorubicin to maintain a resistant phenotype. For studies examining the response to doxorubicin, MCF-7/^{DOX} cells were cultured in the absence of doxorubicin for 3 to 5 days prior to the start of the study. MCF-7/^{WT} cells were cultured in DMEM (4.5 g glucose, 110 mg sodium pyruvate) supplemented with 10% v/v FBS and 10 µg/ml insulin. The *in vitro* toxicity of doxorubicin, silk nanoparticles and doxorubicin-loaded silk nanoparticles was determined by plating 1.9×10^4 cells/cm² in 96-well plates. Cultures were allowed to recover for 24 h. Next, silk nanoparticles or doxorubicin were added to the wells, and cell viability was determined after a 3-day incubation period using (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as detailed elsewhere.²⁰ To study the long-term effect of doxorubicin-loaded silk nanoparticles on breast cancer cells, the cells were treated with 1 µg/ml of doxorubicin or 25 µg/ml silk nanoparticles ± a doxorubicin loading of 1 µg/ml. The cultures were exposed to the treatment for 6 days without a medium change. The cellular response was determined using Alamar Blue

(Invitrogen, Grand Island, NY, USA) as detailed previously.²⁰ Where indicated, the cultures were kept in a low oxygen tension of 5% using a hypoxic incubator.

In vitro trafficking of silk nanoparticles: Cells were seeded onto glass-bottom culture dishes (MatTek Inc. Ashland, MA, USA) and allowed to recover for > 24 h. Next, the cultures were incubated overnight with sterile 1 mg/ml Alexa Fluor 488 silk nanoparticle conjugates (detailed below). To label the acidic intracellular organelles (i.e., lysosomes), LysoTracker Red (Invitrogen, Grand Island, NY, USA) was used according to the manufacturer's instructions. The cells were imaged at one airy disk with a Leica TCS-SP2 laser scanning confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a heated CO₂ live cell incubator chamber. The data were exported to Image J 1.46r (National Institute of Health, USA) for contrast enhancement and assembly of co-localization images.

Preparation and characterization of silk nanoparticles: Bombyx mori fibroin was extracted from cocoons using either a 5 min, 20 min or 60 min extraction time and processed as previously described²⁴ to generate a 5% (wt/v) silk solution. Next, the silk solution was added drop wise (150 μ l/drop) to acetone, maintaining >75% v/v acetone volume. Precipitated silk was then centrifuged at 100,000 $\times g$ for 30 min, the supernatant was aspirated, and the pellet was re-suspended in ddH₂O, vortexed and subsequently sonicated twice for 30 sec at 30% amplitude with a Branson Digital Sonifier 450 (Branson Ultrasonics, Danbury, CT, USA). The centrifugation, washing and re-suspension steps of the silk nanoparticle preparation were repeated at least twice more (Figure 1a). After the final spin, the nanoparticle suspension was progressively filtered using 5- μ m, 0.45- μ m and 0.22- μ m PVDF filters to yield a uniform silk nanoparticle suspension. The particles were subjected to particle analysis as detailed below and stored at 4°C until use.

Nanoparticle characterization: Nanoparticle size distribution was determined by dynamic light scattering (DLS) using a Beckman Coulter Delsa Nano analyzer (Beckman Coulter Inc., Danvers, MA, USA). Particle size distributions were calculated using the Delsa Nano software package and cumulants analysis. The zeta potential of the silk nanoparticles was also determined with the Delsa Nano set up. To determine the nanoparticle yield, a standard curve of absorbance to mass was generated. First, a silk nanoparticle standard curve over a broad range of absorbances was generated. Next, samples were dried at 60°C, and the dry mass of the silk nanoparticles was determined. By determining the amount of silk that was added to the acetone, the final volume and the absorbance of silk nanoparticles, the yield and the concentration of the final silk nanoparticle preparation could be determined. Scanning electron microscopy (SEM) was used to generate qualitative data on the silk nanoparticles. To minimize silk nanoparticle aggregation during the drying process, they were diluted in ultrapure H₂O, spread on silicon wafers, frozen in the gaseous phase of N₂ and dehydrated by freeze-drying. The samples were subsequently sputter coated with a thin coat of platinum/palladium and imaged with a Zeiss Supra 55VP field emission scanning electron microscope (Carl Zeiss, Oberkochen, Germany).

Silk nanoparticles: Loading and release of doxorubicin: The loading characteristics of the silk nanoparticles were determined using either 500 μ g or 125 μ g of silk nanoparticles. The ability of the silk nanoparticles to adsorb doxorubicin was evaluated by using a broad range of drug concentrations (10 – 125 μ g) (N.B. studies performed with ddH₂O). Following a 20 h adsorption period at room temperature under dynamic conditions (20 revolutions per minute), the particles were pelleted using a 40 min centrifugation at 20,000 $\times g$. The amount of drug remaining in solution was determined by measuring doxorubicin-associated fluorescence (excitation 480 nm, emission 590 nm). The process of loading the silk nanoparticles with doxorubicin for the subsequent release studies is detailed below.

pH-dependent release of doxorubicin from silk nanoparticles: A total of 500 μg of the silk nanoparticles was loaded with 20 μg doxorubicin as detailed above. Next, the silk nanoparticle suspension was centrifuged at $20,000 \times g$ for 40 min, yielding a pink pellet. To demonstrate that all samples had been successfully loaded (loading efficiency >96%), doxorubicin-associated fluorescence of the supernatant was determined. The doxorubicin-loaded silk nanoparticle pellet was resuspended in 0.5 ml of phosphate buffer at pH 4.5, 6.0 or 7.4. The samples were loaded into a Slide-A-Lyzer Mini dialysis cartridge (MWCO 3,500 g/mol; Thermo Scientific, Waltham, MA, USA), which was inserted into a 1.5-ml receiver chamber containing 1 ml of buffer at the indicated pH. At the indicated time points, doxorubicin-associated fluorescence in the receiver chamber was monitored, and fresh buffer was added, ensuring that sink conditions were maintained throughout the study.

Labeling silk nanoparticles with Alexa Fluor 488: A total of 2 mg of the silk nanoparticles were resuspended in a 0.2 M NaHCO_3 pH 8.3 buffer to yield a final concentration of 1 mg/ml silk nanoparticles in 0.1 M buffer. Next, 1 mg of Alexa Fluor 488 carboxylic acid succinimidyl ester (Invitrogen, Grand Island, NY, USA) was dissolved in dimethylsulfoxide, and 100 μl of the solution was added to the silk nanoparticles and allowed to react for 4 h at room temperature in the dark while stirring. Following the conjugation of Alexa Fluor 488 to the silk nanoparticles, the suspension was centrifuged, and the pellet was washed repeatedly with acidified water (pH 4.6) to remove unbound dye. The silk nanoparticles were washed until no free dye could be detected in the supernatant. Finally, the particles were dialyzed against ddH_2O for 48 h and processed for *in vitro* cell culture studies as detailed above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Figure 1.

Manufacture and characterization of silk nanoparticles. **(A)** A flow diagram depicting the steps required to generate silk nanoparticles. **(B)** Qualitative characterization of the silk nanoparticles by scanning electron microscopy (scale bars 200 nm). **(C)** Size and surface charge of the silk nanoparticles determined by dynamic light scattering and zeta potential measurements.

Figure 2. Ability of the silk nanoparticles to bind and release doxorubicin using pH as a stimulus. **(A)** Binding isotherm of doxorubicin to the silk nanoparticles. **(B)** pH-dependent release of doxorubicin during the first 24 h and subsequent 6 days **(C)**. (Significant differences were determined with ANOVA followed by Bonferroni's multiple comparison post hoc test, * $P < 0.05$, ** $P < 0.001$; $\pm$ SD; $n = 3$).

Figure 3. Doxorubicin-loaded silk nanoparticles overcome drug resistance in human breast cancer cells. **(A)** Three-day cytotoxicity of the silk nanoparticles in human breast cancer cell lines under normoxic and hypoxic culture conditions ($\pm$ SD; $n = 3$), **(B)** Cytotoxicity of the silk nanoparticles, free drug and doxorubicin-loaded silk nanoparticles in sensitive (MCF-7^{WT}) and anthracycline-resistant (MCF-7^{DOX}) breast cancer cells under normoxic and hypoxic culture conditions. (Significant differences were determined with Student's t-test, * $P < 0.05 \pm$ SD; $n = 3$).

Figure 4.

Lysosomotropic delivery of the silk nanoparticles in human breast cancer cells. **(A)** Live cell confocal images of MCF-7/^{WT} exposed to: (i) green fluorescent silk nanoparticles (SNPs), or (ii) LysosTracker red to stain lysosomes, and (iii) merged respective green and red fluorescent channels. **(B)** Magnified images of (i) the silk nanoparticles (SNPs) in endocytic vesicles, (ii) stained lysosomes, and (iii) merged images of the respective fluorescent channels. Arrowhead demonstrates lysosomal accumulation of silk nanoparticles. **(C)** Live cell confocal images of MCF-7/^{DOX} exposed to the silk nanoparticles (SNPs) resulting in endocytic labeling. Scale bars 30 μ m.

Figure 5.
A schematic representation of silk nanomedicines. pH-dependent release of doxorubicin from a silk nanoparticle. Diagram not drawn to scale.