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Modulation of Vincristine and Doxorubicin Binding and Release from Silk Films

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Abstract

Sustained release drug delivery systems remain a major clinical need for small molecule therapeutics in oncology. Here, mechanisms of small molecule interactions with silk protein films were studied with cationic oncology drugs, vincristine and doxorubicin, with a focus on hydrophobicity (non-ionic surfactant) and charge (pH and ionic strength). Interactions were primarily driven by charge interactions between the positively charged drugs and the negatively charged groups within the silk films. Exploiting chemical modifications of silk further modulated the drug interactions in a controlled fashion. Increasing anionic side groups via carboxylate- and sulfonate-modifications of tyrosine side chains in the silk protein using diazonium coupling chemistry, increased drug binding and altered drug release. The effects of silk film protein crystallinity, beta sheet content, on drug binding and release was also explored. Lower crystallinity supported more rapid drug binding when compared to higher crystalline silk films. The drug release kinetics were governed by the protonation state of vincristine and doxorubicin and were tunable based on silk crystallinity and chemistry. These studies depict an approach to characterize small molecule-silk protein interactions and methods to tune drug binding and release kinetics from this protein delivery matrix.

Graphical Abstract

Keywords

Cancer; doxorubicin; vincristine; controlled release; biomaterials; silk fibroin

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Introduction

Cancer remains a leading cause of death world-wide [1]. Cancer treatments generally consist of surgery, radiation and systemic delivery of chemotherapy drugs. Chemotherapy drugs target different components involved in cell proliferation, resulting in cell death. However, these drugs are non-specific and affect all rapidly proliferating cells including healthy cells and organ functions [2]. These off-target effects come with significant secondary side effects, including nausea, vomiting, diarrhea [3, 4], with more serious side effects including ototoxicity, myelosuppression, nephrotoxicity, cardiotoxicity, peripheral neuropathy and secondary malignancies [5, 6, 7, 8, 9, 10].

Enhanced delivery techniques are a potential way to reduce chemotherapy-related toxicity while enhancing the treatment of difficult to treat cancers. Toward this goal, liposomal delivery vehicles are used for systemic delivery of doxorubicin, daunorubicin and vincristine, including Doxil®, DaunoXome® and Marqibo®, respectively. Liposomal delivery of doxorubicin increased vascular retention while significantly decreased drug distribution in organs [11, 12]. Liposome formulations also increase drug payload to the tumor site via enhanced permeability and retention [13] and significantly decreased organ toxicity [14, 15, 16]. The one clinically used, locally administered chemotherapy release system, Giadel® wafer containing carmustine, is used for the treatment of glioblastoma and has been shown to increase patient survival [17, 18, 19]. The Giadel® wafer system relies on diffusion and material degradation for the release of carmustine. However, there remains a major need for improved delivery systems for chemotherapy agents to increase the range of drugs delivery, decrease systemic toxicity and increase treatment effectiveness.

Silk fibroin protein from silkworm cocoons (referred to hereon as silk) has been studied for the release of a variety of therapeutic small molecules [20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32]. Chemotherapy agents such as doxorubicin and platinum-based drugs reversibly bind to silk resulting in sustained release *in vitro* and *in vivo* [26, 27, 28, 29, 30, 31, 32]. New options to modify and tune the binding and release of drugs from silk would enhance the utility of this protein material in drug delivery. Silk consist of three main proteins: heavy chain, light chain and fibrohexamerin [33, 34]. Silk fibroin heavy chain contributes approximately 93% of the isolated solid silk weight. Structurally, it is a high molecular weight amphiphilic protein composed of 12 highly repetitive, hydrophobic domains (mostly glycine-alanine repeats) separated by 11 short amorphous domains [35]. Silk is a useful drug delivery candidate due to its chemical structure which has potential to interact with hydrophobic, hydrophilic and charged molecules. In addition to the structurally and chemically useful properties, silk is biocompatible, biodegradable, extracted and processed via all aqueous conditions and amendable to injectable, implantable and transdermal applications [36]. Silk also has been used for centuries as sutures and more recently received FDA approval as a surgical mesh (Allergan Inc., Irvine, CA, USA) for use in humans.

Silk has suitable amino acid side chains for chemical modification, which provides a versatile system for tuning drug binding and release. Numerous chemical modification schemes have been developed to introduce multiple chemical groups and to conjugate peptides, proteins, polysaccharides and synthetic polymers to silk [37]. A reaction scheme

using diazonium coupling chemistry has been used to introduce multiple functional groups into silk via an electrophilic aromatic substitution between a diazonium salt and the tyrosine side chains [38]. Silk materials modified via diazonium coupling chemistry to introduce sulfonate groups have been investigated for the sustained release of C-X-C motif chemokine 12 and fibroblast growth factor-2 [39, 40].

Because there has been limited clinical success using diffusion and degradation based release mechanism, investigation of alternative polymer-based delivery vehicles is needed. The versatility of silk-based systems including multiple modes of delivery (e.g., injectable, implantable), easily tuned material crystallinity to alter diffusion, and amenability for chemical modifications makes silk a potential biomaterial to address these current limitations. We have previously reported on the release of doxorubicin from silk films [27, 28]. The present study investigated the mechanisms by which doxorubicin and vincristine, two cationic chemotherapeutic agents, bind to silk films. The silk was chemically modified with anionic functional groups to alter drug interactions to silk films. The mechanisms of drug-silk interaction were probed using solution pH and ionic strength to investigate ionic interactions, and a non-ionic surfactant to investigate hydrophobic interactions. Additionally, two post-processing treatments were utilized to investigate the effect of silk film crystallinity on drug binding kinetics. This work allowed for mechanistic insight into the interplay between diffusion and drug-silk material interactions on drug binding. Furthermore, the drug release studies provided translationally relevant information on the silk parameters tested which can be used to further optimize and tune the release of small molecules from silk materials for the treatment of cancer and other diseases.

Materials and Methods

Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Doxorubicin-HCl and vincristine sodium sulfate were purchased from LC Laboratories (Woburn, MA). Deionized water used for silk processing and solution preparation was obtained via a PicoPure® water purification system (Hydro Service and Supplies, Durham, NC). Cocoons produced by *Bombyx mori* silkworms were obtained from Tajima Shoji Co (Yokohama, Japan).

Silk solution preparation

Silk solution was prepared from *B. mori* silk worm cocoons as previously described [41]. Briefly, cocoons were cut into approximately 1 cm x 1 cm pieces and boiled in 0.02 M Na₂CO₃ solution for 30 minutes to remove the sericin proteins. The boiled silk fibers were washed 3 times with deionized water and allowed to dry overnight in a fume hood. Five grams of dried silk fibers were dissolved in 20 mL 9.3 M LiBr solution at 60°C followed by dialysis (Pierce 3.4 kDa MWCO dialysis cassette; Fisher Scientific, Pittsburg, PA) against deionized water for 2 days. The aqueous silk solution (6–7%) was stored at 4°C until use.

Diazonium coupling reaction with silk

Modification of silk via diazonium coupling was performed as previously described [38]. Briefly, after dialyzing the silk solution for 1.5 days in deionized water, dialysis was performed for 1 day (2 solution changes) against 0.1 M borate buffer containing 150 mM NaCl at pH 9 (BupH borate buffer pak; Pierce, Woburn, MA). The resulting 8% silk solution was immediately processed for diazonium coupling. A 1.25 mL aniline solution, 0.2 M 4-aminobenzoic acid acetonitrile solution or 0.2 M 4-sulfanilic acid water solution, was cooled on ice for 15 minutes. Equal volumes of 1.6 M aqueous p-toluenesulfonic acid and 0.8M NaNO₂ were mixed and cooled on ice for 15 minutes. Equal volumes of each pre-cooled solution were combined and allowed to react on ice for 30 minutes to form the diazonium salt. To 2 mL silk solution, 0.5 mL diazonium salt was added and allowed to react on ice for 30 minutes. The reacted silk solution was dialyzed against deionized water for 2 days to remove unreacted components and salts. The dialyzed modified silk solutions was lyophilized. The dried modified silk was stored at 4°C until use. Quantification of tyrosine modification was performed using the azo bond peak at ~328 nm as previously described [38].

Silk film production

Silk solutions were prepared at 40 mg/mL. Fifty eight microliters of the silk solution was pipetted onto 7.5 mm x 7.5 mm polydimethylsiloxane (Sylgard 184, Dow Corning, Corp. Midland, MI) molds and allowed to dry overnight. Dried silk films were postprocessed via water-vapor annealing at room temperature for 16–24 hours or autoclaving at 121°C for 20 minutes to induce low and high crystallinity and render the films insoluble. The weight of at least four films from two different batches were measured after post-processing to confirm equal silk film masses across groups.

Fourier transform infrared spectroscopy analysis

Fourier transform infrared spectroscopy (FTIR) was performed with a Jasco FT/IR6200 spectrometer (JASCO, Tokyo, Japan) equipped with a MIRacle™ attenuated total reflection (ATR) Ge crystal cell in reflection mode. For each measurement, 32 scans of 4 cm⁻¹ resolution were co-added and Fourier transformed using a Blackman-Harris apodization function. The secondary structure of the silk films was characterized between 1585 and 1710 cm⁻¹ representing the amide I region. The amide I region was deconvoluted using Opus 5.0 software (Bruker, Billerica, MA) as previously described [42, 43, 44]. Briefly, the spectra were normalized and baseline corrected between 1750–1150 cm⁻¹ followed by Fourier self-deconvolution (FSD) between 1720–1585 cm⁻¹ using a bandwidth of 27.5 cm⁻¹, noise reduction of 0.3 and Lorentzian line shape. The FSD spectra were baseline corrected between 1710–1585 cm⁻¹ and curve-fitted to measure the relative areas of the Amide I region structures assuming the C=O stretch is the same for all secondary structures [43]. Peak positions were first defined using the second derivative of the original spectra and a local least squared analysis performed. The peaks were then held constant and a Levenberg Maquardt algorithm was used to optimize the peak width and height. This allowed for the resulting curve fit to closely resemble the initial FSD spectrum. Peak positions were defined as follows [42, 43, 44]: 1615–1630⁻¹ and 1695–1705 cm⁻¹ as β -sheet structure; 1631–1655

cm⁻¹ as random-coil structure; 1650–1660 cm⁻¹ as α -helical bands; and 1660–1695 cm⁻¹ as β turns.

Drug binding studies

Multiple types of drug binding studies were performed to characterize the binding mechanisms, binding kinetics and equilibrium adsorption. For binding mechanisms characterization, drug solutions were prepared at 200 μ g/mL in varying aqueous media to evaluate the contribution of hydrophobic and charge interaction on drug binding following a previously published work [45]. To evaluate hydrophobic interaction, a non-ionic surfactant, polysorbate 80 at 0.1% (v/v), was used. To evaluate charge interaction, both pH and ionic strength were varied (pH 3 and 15 mM NaCl or 150 mM NaCl). Unmodified and modified silk films were placed into 1 mL or 4 mL of aqueous drug media, respectively, for 4 days. All films were post-processed via water vapor annealing. The drug binding was determined by:

$$\text{Drug bound } (\mu\text{g}) = \left[\text{initial drug concentration } \left(\frac{\mu\text{g}}{\text{mL}} \right) - \text{final drug concentration } \left(\frac{\mu\text{g}}{\text{mL}} \right) \right] \times \text{volume (mL)}$$

Where the final drug concentration was determined via UV/vis absorbance and standard curves. An additional drug binding study with varying pH was performed in a similar manner using unmodified, water vapor annealed silk films.

To characterize the binding kinetics, water vapor annealed and autoclaved silk films were evaluated. Silk films (unmodified, carboxylate and sulfonate) were placed into 1 mL or 4 mL of 200 μ g/mL drug solution in water, respectively. The final or unbound concentration was monitored periodically and the solution placed back into the tubes. To characterize the equilibrium adsorption, water vapor annealed and autoclaved silk films were evaluated. Silk films (unmodified, carboxylate and sulfonate) were placed into 1 mL of drug solution with varying drug concentration. After 1 month, the remaining drug concentration was quantified and the bound drug mass determined.

Drug Release studies

Films from the kinetic adsorption studies were evaluated for drug release. Silk films were placed in 1 mL PBS, pH 7.4 (Life Technologies, Grand Island, NY) at 37°C. At periodic time points, the UV/Vis absorbance was measured to determine the mass of released drug. The PBS solution was completely replaced with new PBS.

Ultraviolet/visible light spectroscopy

Ultraviolet/visible (UV/Vis) light spectroscopy (SpectraMax M2 spectrophotometer; Molecular Devices, Sunnyvale, CA) was used to determine the drug solution concentration at 298 nm and 482 nm for vincristine and doxorubicin, respectively. All measurements were performed in UV-transparent 96-well plates using 200 μ L media. Standard curves of drug concentration versus absorbance were developed to quantify the unknown drug concentration in solution. Standard curves in all media conditions were generated (water, PBS, pH 3 and 0.1% Tween) and were similar.

Statistics

Three independent experiments with three separated samples were performed for each study. Data are expressed as mean $\pm$ standard deviation (SD) of three independent experiments for drug binding mechanism, drug loading and release studies. Data are expressed as mean $\pm$ standard deviation (SD) of three samples from one experiment for kinetic adsorption and equilibrium adsorption due to time variation and drug concentration variation across independent experiments, respectively. Statistical analysis was performed only on data combined from three independent experiments. Statistical significance was determined by student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using SPSS 22.0 software (IBM, Armonk, NY). Significance was determined at $P < 0.05$.

Results and Discussion

Modified Silk and Silk Film Characterization

To study drug-silk interactions three silk formats were evaluated: (1) unmodified silk, (2) COOH-modified silk and (3) SO₃H-modified silk. The silk modifications were chosen to increase the content of negative charge groups on silk. The degree of azo-modification was evaluated spectrophotometrically by UV/Vis absorption as previously characterized [38]. Under the reaction conditions used for these experiments, comparable levels of tyrosine modifications were achieved, 26% and 28% for the carboxylate and sulfonate modification, respectively (Supplementary Figure 1). The silk film chemistry groups are noted as: unmodified, carboxylate (carboxylate-modified) or sulfonate (sulfonate-modified).

In previous work, silk crystallinity significantly altered small molecule release from silk films and drug encapsulation reservoirs [21, 23, 28]. Therefore, two post-processing methods were also investigated to alter the silk film crystallinity. Quantification of the silk film secondary structure was determined by FTIR (Supplementary Figure 2). Fourier self-deconvolution of FTIR spectra has been developed as an excellent technique to characterize protein secondary structure and crystallinity that closely estimates the results from x-ray diffraction experiments [46, 47]. Characterization of silk crystallinity has previously been described for water-vapor, autoclave and methanol annealed silk films [43, 44, 48]. Carboxylate and sulfonate modifications altered the secondary structure of the water-vapor annealed silk films (Supplementary Figure 2A, curve a–c). For the water-vapor annealed silk films, the β -sheet content was 30%, 33% and 30% and random coil content was 44%, 36% and 40% for unmodified, carboxylate and sulfonate materials, respectively (Supplementary Figure 2B). Chemical modifications of silk induced minimal changes in the silk secondary structure for the autoclaved annealed films (Supplementary Figure 2A, curve d–f). For the autoclaved silk films, the β -sheet content was 48%, 45% and 43% and random coil content was 25%, 26% and 29% for unmodified, carboxylate and sulfonate materials, respectively (Supplementary Figure 2B). From here on, the silk film post-processing groups will be noted as: and low crystallinity (water-vapor annealed) or high crystallinity (autoclaved).

Silk-Drug Binding

Next, we sought to determine the mechanisms of drug binding to silk films. Previously, the chemotherapeutic drugs cisplatin, doxorubicin and crizotinib, were successfully loaded into silk materials via adsorption in water [26, 28, 29, 49]. In the present work, we employed the same adsorption mechanism for doxorubicin and vincristine binding to silk films. Both drugs bound to silk as seen via depletion from solution (Figure 1). Increasing the anionic groups in silk films statistically increased vincristine and doxorubicin binding when compared to unmodified silk films (Figure 1). The carboxylate and sulfonated silk films had comparable doxorubicin levels, while the sulfonate silk had increased vincristine loading when compared to the carboxylate modification.

After confirming that doxorubicin and vincristine bind to unmodified and modified silk films, the mechanism of drug binding was investigated. Low crystalline silk films were used for these studies because there were no differences in drug binding between low crystalline and high crystalline silk films (Figure 1). A conventional mechanism proposed for small molecule binding to silk films is hydrophobic interactions with the hydrophobic, GAGAGS and GAGAGY domains of silk. Therefore, two modes of binding were probed: hydrophobic and charge interactions. A non-ionic surfactant, polysorbate 80 (PS80), was included in the binding media at a concentration of 0.1% (w/v) to compete with these hydrophobic interactions. A ten-fold higher concentration was initially evaluated, however significant drug degradation was observed (data not shown) and was attributed to autooxidation of polysorbate 80 in solution giving rise to hydroperoxide formation and drug degradation [50]. At 0.1% (w/v) PS80, no observable drug degradation occurred within the four days of the experiment. Inclusion of 0.1% (w/v) PS80 in the binding media did not significantly alter drug binding (Figure 2 A, B).

To probe charge interactions, the initial pH and ionic strength of the binding media was varied. At pH 3, binding decreased significantly for all film groups (Figure 2 C, D). For unmodified and carboxylate films, this decrease was to <5% and <7% of drug binding in water, respectively. For the sulfonate silk films, this decrease was approximately 50% of the binding in water. At pH 3, the sulfonate groups are still deprotonated allowing for drug interactions under these acidic conditions. To further probe pH effects on drug binding, a pH titration study was performed using unmodified, low crystalline silk films (Supplementary Figure 3 A, B). At pH, 2, 3 and 4, minimal drug binding was observed, while increasing the pH resulted in a pH-dependent increase in drug binding. To confirm that the silk films exposed to acidic conditions retained drug binding capacity, the films were then placed into drug solutions at approximately pH 6. These films were able to bind the drugs at pH 6 which showed reversible drug binding inhibition occurred under acidic conditions (Supplementary Figure 3 C, D). This reversibility suggested that inhibited binding at low pH was a non-destructive, charge-driven process.

The ionic strength of the binding media also altered drug binding. For doxorubicin, all films exposed to 150 mM NaCl media exhibited a statistical decrease in drug binding (Figure 2 E). However, doxorubicin binding in 15 mM NaCl media was statistically decreased for the unmodified films only (Figure 2E). For vincristine, increasing NaCl concentration decreased drug binding in a concentration-dependent manner (Figure 2 F). This combined data from

non-ionic surfactant, pH and ionic strength compositions of the binding media strongly suggest that charge interactions are the primary mediators of drug binding to the modified and unmodified silk films.

Drug Binding Kinetics

To explore the effect of chemistry and crystallinity on drug binding rates, kinetic adsorption studies were performed using the unmodified, carboxylate and sulfonate silk films with low and high crystallinity. Solution UV/Vis absorbance was evaluated at each time point and the difference between initial and final solution concentrations was used to quantify drug binding. Initially, rapid drug binding was observed (Figure 3, 4) and this was generally followed by slow drug binding, most notably for the higher crystalline silk films. For low crystallinity silk films rapid doxorubicin binding commenced after approximately six hours (Figure 3, **white shapes**); thereafter very little additional binding occurred for the unmodified silk films. However, carboxylate and sulfonate, low crystalline silk films exhibited additional binding after the initial rapid binding phase (Figure 3 B, C, **white shapes**). Doxorubicin binding to all high crystalline silk films was statistically slower than low crystalline silk films when compared across the same chemical modification (Supplementary Table 1) and did not plateau by day 13 (Figure 3 A to C **grey circles**). Vincristine exhibited similar binding behavior as doxorubicin (Figure 4). There was an initial rapid vincristine binding phase, which was statistically faster for low crystalline films compared to high crystalline films (Supplementary Table 2). Interestingly, sulfonate silk films of both low and high crystallinity exhibited similar vincristine binding rates after the initial rapid binding phase (Figure 4 C). In summary, drug binding to all silk film groups exhibited an initial rapid phase. The rapid initial binding was likely due to rapid diffusion and access to surface charge groups. The slower binding phase was likely due to less accessible charge sites within the silk films, most notably in the higher film crystallinity. This effect was also observed during the initial binding phase, where binding to low crystalline films was faster than for high crystalline films.

Equilibrium Adsorption

Equilibrium adsorption studies were performed to determine partitioning between the solid silk films and aqueous solution. The mass of silk within the system was held constant, while increasing drug concentration in a fixed solution volume was evaluated (Figure 5, 6). Drug amounts less than the maximum binding capacity were evaluated to determine the partitioning between the solid silk film and solution. The slope of the drug bound versus total drug in the region less than the maximum drug binding capacity (linear portion) gives the percentage of drug bound. Nearly complete doxorubicin binding was observed at drug amounts less than the maximum binding capacity as evidenced by slopes between 0.95 and 0.98 for drug bound versus total drug (Supplementary Table 3). No statistical differences were observed for the doxorubicin slopes between film chemistries or crystallinities. For vincristine, the slope ranged from 0.77 to 0.95 for the different film formats evaluated (Supplementary Table 4). No statistical differences were observed when comparing low crystallinity silk films with different chemistries. Carboxylate and sulfonate, high crystallinity silk films exhibited significantly higher slopes than the unmodified high

crystallinity silk films for vincristine adsorption. For both doxorubicin and vincristine, there were no statistical differences in bound drug at the highest two or three total drug amounts.

Doxorubicin and Vincristine Release from Silk Films

In vitro release of doxorubicin and vincristine from silk films was evaluated. Drug-loaded silk films from the kinetic adsorption studies were used. Therefore, loading varied depending on the silk film chemistry (Figure 1). Doxorubicin exhibited sustained release through the experimental time course (Figure 7). Between 45% and 60% of the drug remained in the silk film after 28 days of release depending on the chemical modification and post-processing technique used. One release study was also evaluated through 72 days and 74 days for low crystallinity and high crystallinity silk films, respectively (Supplementary Figure 4). Continuous doxorubicin release was observed, suggesting that complete drug release may be achieved. By taking the slope of the release data between 40 days and 72 days or 74 days, complete doxorubicin release was projected to take (1) 60 weeks and 63 weeks for low crystallinity and high crystallinity unmodified silk films, (2) 68 and 64 weeks for the low crystallinity and high crystallinity carboxylate silk films and (3) 71 weeks and 72 weeks for low crystallinity and high crystallinity sulfonate silk films, respectively. Based on this projection data, little difference in release duration between low crystalline and high crystalline silk films can be predicted. These release studies were performed using PBS which does not completely recapitulate the *in vivo* environment. The presence of proteolytic enzymes *in vivo* capable of degrading silk materials, potentially destabilizing drug-silk interactions, may decrease the release duration. However, it has been previously shown that the short term release (within one month) of doxorubicin from silk films is minimally altered in the *in vivo* environment [27]. In particular, doxorubicin release only increased during the first four days *in vivo* which may be due to proteolysis or the differences in ionic composition between PBS and body fluids. After the initial release period, the release rate of doxorubicin from silk films *in vitro* and *in vivo* was similar.

Varying the crystallinity of the silk films altered the release of doxorubicin (Table 1). For both the unmodified and carboxylate groups, the low crystalline silk film day 1 (D1) mass release was statistically higher than that of the high crystalline silk film. The low crystalline and high crystalline sulfonate silk films exhibited D1 mass release. However, only the unmodified low crystalline and high crystalline silk films had statistically different cumulative day 28 (D28) mass release. On a percent release basis, unmodified silk films exhibited statistically higher doxorubicin release on D1 and through D28 when comparing high crystalline silk films to low crystalline silk films. Only the cumulative D28 release was statistically higher for the low crystalline compared to the high crystalline when using sulfonate silk films. No differences were observed in percent release when comparing the two different crystalline groups of the carboxylate silk films and sulfonate silk films, respectively.

Increasing the concentration of anionic groups on silk altered doxorubicin release from silk films (Table 1). Low crystallinity, carboxylate silk films had statistically lower D1 and cumulative D28 mass release when compared to unmodified silk films with the same postprocessing treatment; while sulfonate silk films had statistically higher cumulative D28

release when compared to unmodified silk films. High crystallinity, sulfonate silk films had statistically higher D1 and cumulative D28 mass release when compared to unmodified silk films with the same post-processing treatment. No differences were observed in mass release between high crystallinity, carboxylate silk films and high crystallinity, unmodified silk films. Both the carboxylate and sulfonate silk film groups had significantly decreased percent release compared to unmodified silk films owing to the significantly higher drug loading. These materials would likely result in a much longer release duration than the unmodified silk films owing to the increased drug loading and decreased percent release. One potential method to further increase the release rate may be to further decrease silk film crystallinity. Further decreasing the crystallinity may be advantageous to achieve release durations comparable to intravenous chemotherapy administration cycles. Tuning the crystallinity of water-vapor annealed, unmodified silk films has been well characterized [44].

Vincristine exhibited rapid and nearly complete release by day 10 (Figure 8); after day 21, vincristine in the release media was below the sensitivity of the assay for all groups. Since a significant amount of doxorubicin remained bound to the silk films after 28 days of release, this suggests that doxorubicin binds more strongly to silk films compared to vincristine once submerged into a PBS solution. This difference in release between vincristine and doxorubicin may be due to the differences in pKa. The reported pKa of doxorubicin ranges from 8.3 to 9.5 [51, 52, 53] compared to 5.0 and 7.4 for vincristine [54]. In PBS at pH 7.4, a portion of vincristine is in the deprotonated state, resulting in loss of charge interaction and rapid release. For doxorubicin, other silk interactions have been proposed and may play a secondary role in interaction and stabilization after the initiation doxorubicin binding [28].

Film crystallinity significantly affected the D1 vincristine release for the unmodified silk films where increased crystallinity decreased D1 vincristine mass and percent release (Table 2). However, for the cumulative D28 mass release, there was no difference between post-processing treatment groups containing the same silk chemistry. The D1 mass release for both the unmodified and carboxylate, high crystalline silk films were statistically lower when compared to the unmodified and carboxylate, low crystalline silk films, respectively. However, no differences in percent release were observed between low and high crystallinity silk films of identical silk chemistry. The most dominant trend in vincristine release was that increased drug loading increased the amount of drug release at each time point, which was achieved by introducing carboxylate and sulfonate groups into the silk.

Conclusions

The mechanism of doxorubicin and vincristine binding to silk films was determined. The driving force for binding to silk was via electrostatic interactions between the cationic group(s) on the drugs and the anionic amino acid side groups on silk. This insight was achieved by altering the pH and ionic strength of the drug solution. By increasing the anionic functional groups on silk, increased drug binding was achieved. Drug binding capacity was independent of silk film crystallinity while drug binding kinetics were significantly affected by silk film crystallinity. By altering silk film crystallinity and chemical composition altered drug release was achieved. Further decreasing the silk film

crystallinity by water-vapor annealing at reduced temperatures or for a shorter time periods may alter the release kinetics by loosening the silk network and increasing diffusion. In the future, other chemical modifications (e.g., amine groups) can be explored to bind additional drugs to expand the binding capabilities of silk. These results establish a foundation for developing more chemically and physically advanced silk drug delivery systems for use in treatments of cancer and other diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Drug loading of silk films

(A) Doxorubicin and (B) vincristine loading in unmodified (silk), carboxylate (silk modified via diazonium coupling chemistry to increase carboxylate content) and sulfonate (silk modified via diazonium coupling chemistry to introduce sulfonate groups) low crystallinity and high crystallinity silk films. Data represent the mean $\pm$ S.D. from three independent experiments each with three separate samples. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ between unmodified and carboxylate or sulfonate silk films within the same postprocessing treatment; § $p < 0.05$, §§ $p < 0.01$ between sulfonate and carboxylate silk films with the same post-processing treatment.

Figure 2. Doxorubicin and vincristine binding to low crystallinity silk films under varying solution conditions

Effect of (A and B) polysorbate 80, (C and D) pH and (E and F) ionic strength on (A, C and E) doxorubicin and (B, D and F) vincristine binding to unmodified (silk), carboxylate (silk modified via diazonium coupling chemistry to increase carboxylate content) and sulfonate (silk modified via diazonium coupling chemistry to introduce sulfonate groups) silk films. Data represent the mean $\pm$ S.D. from three independent experiments each with three separate samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between unmodified and carboxylate or sulfonate silk films within the same post-processing treatment.

Figure 3. Kinetic adsorption of doxorubicin on silk films

Doxorubicin binding to (A and D) unmodified (silk), (B and E) carboxylate (silk modified via diazonium coupling chemistry to increase carboxylate content) and (C and F) sulfonate (silk modified via diazonium coupling chemistry to introduce sulfonate groups) low crystallinity (white symbols) and high crystallinity (grey symbols) silk films. The experiments were performed for (A–C) one week (low crystallinity) or two weeks (high crystallinity); (D–F) shows the data for the first 8 hours. Data represent the mean $\pm$ S.D. from three separate samples.

Figure 4. Kinetic adsorption of vincristine on silk films

Vincristine binding to (A and D) unmodified (silk), (B and E) carboxylate (silk modified via diazonium coupling chemistry to increase carboxylate content) and (C and F) sulfonate (silk modified via diazonium coupling chemistry to introduce sulfonate groups) low crystallinity (white symbols) and high crystallinity (grey symbols) silk films. The experiments were performed for (A–C) one week (low crystallinity) or two weeks (high crystallinity); (D–F) shows the data for the first 8 hours. Data represent the mean $\pm$ S.D. from three separate samples.

Figure 5. Equilibration adsorption of doxorubicin to silk films

Doxorubicin binding to (A, C and E) low crystallinity and (B, D and F) high crystallinity unmodified (silk), carboxylate (silk modified via diazonium coupling chemistry to increase carboxylate content) and sulfonate (silk modified via diazonium coupling chemistry to introduce sulfonate groups) silk films. Data represent the mean $\pm$ S.D. from three independent experiments, N.D.: no differences between groups.

Figure 6. Equilibration adsorption of vincristine to silk films

Vincristine binding to (A, C and E) low crystallinity and (B, D and F) high crystallinity unmodified (silk), carboxylate (silk modified via diazonium coupling chemistry to increase carboxylate content) and sulfonate (silk modified via diazonium coupling chemistry to introduce sulfonate groups) silk films. Data represent the mean $\pm$ S.D. from three independent experiments, N.D.: no differences between groups.

Figure 7. Release of doxorubicin from silk films

Cumulative mass release of doxorubicin from (A) low crystallinity and (B) high crystallinity silk films. Cumulative percent release of doxorubicin from (C) low crystallinity and (D) high crystallinity silk films. Silk films were either unmodified (silk), carboxylate (silk modified via diazonium coupling chemistry to increase carboxylate content) and sulfonate (silk modified via diazonium coupling chemistry to introduce sulfonate groups). Data represent the mean $\pm$ S.D. from three independent experiments each with three separate samples.

Figure 8. Release of vincristine from silk films

Cumulative mass release of vincristine from (A) low crystallinity and (B) high crystallinity silk films. Cumulative percent release of vincristine from (C) low crystallinity and (D) high crystallinity silk films. Silk films were either unmodified (silk), carboxylate (silk modified via diazonium coupling chemistry to increase carboxylate content) and sulfonate (silk modified via diazonium coupling chemistry to introduce sulfonate groups). Data represent the mean $\pm$ S.D. from three independent experiments each with three separate samples.

Table 1

Summary of Doxorubicin Release from Silk Films

Group	Low Crystallinity		High Crystallinity	
	D1	Cumulative D28	D1	Cumulative D28
Mass Release (μg)				
Unmodified	46 ± 2	87 ± 3	$26 \pm 1^{\dagger\dagger\dagger}$	$62 \pm 7^{\dagger\dagger}$
Carboxylate	$33 \pm 3^{***}$	$77 \pm 4^*$	$25 \pm 3^{\dagger}$	71 ± 8
Sulfonate	$43 \pm 2^{\S}$	$100 \pm 4^{*,\S}$	$44 \pm 2^{***,\S\S\S}$	$114 \pm 10^{**,\S\S\S}$
Percent Release (%)				
Unmodified	30 ± 1	56 ± 2	$16 \pm 1^{\dagger\dagger\dagger}$	$40 \pm 3^{\dagger\dagger\dagger}$
Carboxylate	$6 \pm 0.4^{***}$	$14 \pm 0.3^{***}$	$5 \pm 0.2^{***}$	$16 \pm 2^{***}$
Sulfonate	$8 \pm 0.4^{***}$	$18 \pm 1^{***,\S\S}$	$9 \pm 1^{***}$	$23 \pm 1^{**,\S\S}$

Data from three independent experiments

* $p < 0.05$ when compared to unmodified within the same post-processing treatment** $p < 0.01$ when compared to unmodified within the same post-processing treatment*** $p < 0.001$ when compared to unmodified within the same post-processing treatment † $p < 0.05$ when comparing high crystallinity to low crystallinity with the same chemistry †† $p < 0.01$ when comparing high crystallinity to low crystallinity with the same chemistry ††† $p < 0.001$ when comparing high crystallinity to low crystallinity with the same chemistry § $p < 0.05$ when comparing sulfonate to carboxylate silk films with the same post-processing treatment §§ $p < 0.01$ when comparing sulfonate to carboxylate silk films with the same post-processing treatment

Table 2

Summary of Vincristine Release from Silk Films

Group	Low Crystallinity		High Crystallinity	
	D1	Cumulative D21	D1	Cumulative D21
Mass Release (μg)				
Unmodified	85 ± 6	113 ± 7	60 ± 11	86 ± 17
Carboxylate	$141 \pm 8^{***}$	$307 \pm 43^{***}$	$121 \pm 7^{***}$	$238 \pm 37^{***}$
Sulfonate	$210 \pm 16^{***, §§§}$	$386 \pm 47^{***}$	$200 \pm 19^{***, §§§}$	$333 \pm 33^{***, §}$
Percent Release (%)				
Unmodified	76 ± 5	101 ± 6	$60 \pm 8^{\dagger\dagger}$	91 ± 8
Carboxylate	$45 \pm 5^{***}$	98 ± 10	$44 \pm 1^{**}$	92 ± 5
Sulfonate	$54 \pm 2^{***}$	100 ± 6	$53 \pm 1^{\$ \$}$	95 ± 1

Data from three independent experiments

* $p < 0.05$ when compared to unmodified within the same post-processing treatment** $p < 0.01$ when compared to unmodified within the same post-processing treatment † $p < 0.05$ when comparing high crystallinity to low crystallinity with the same chemistry $^{\$}$ $p < 0.05$ when comparing sulfonate to carboxylate silk films with the same post-processing treatment $^{\$ \$}$ $p < 0.01$ when comparing sulfonate to carboxylate silk films with the same post-processing treatment