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Doxorubicin-loaded silk films: drug-silk interactions and *in vivo* performance in human orthotopic breast cancer

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Abstract

Breast cancer is the most common of all malignant diseases in women. Systemic chemotherapy provides low clinical benefit for locoregional control of the disease, while localised chemotherapy may provide a therapeutic advantage. In this study, doxorubicin-loaded silk films were directly applied to tumours. Affinity binding studies demonstrated that the adsorption of doxorubicin onto silk was partially dependent on crystallinity. By manipulating silk crystallinity, or β -sheet content, the doxorubicin release rate could be controlled ranging from immediate release to prolonged release over >4 weeks. Following successful *in vitro* studies, the therapeutic impact of doxorubicin-loaded silk films on primary tumour growth and metastasis was assessed in mice using a humanised orthotopic breast cancer model (adenocarcinoma). Both soluble and stabilised silk films loaded with doxorubicin had a significantly greater primary tumour response than the equivalent dose of doxorubicin administered intravenously in the absence of the silk film carrier. In addition to reducing primary tumour growth, stabilised silk films loaded with doxorubicin also reduced metastatic spread and autopsy indicated that these films were not associated with any local or systemic toxicities. Collectively, these results suggest that the future use of this approach for localised chemotherapy is promising.

INTRODUCTION

Breast cancer is the most common of all malignant diseases in women, with one in nine women developing breast cancer during their lifetime. Hormonal status and disease stage dictate the treatment regimen; for early breast cancer, the standard of care includes tumour resection [1]. Over the past 35 years, there has been a shift towards breast conserving surgeries, or lumpectomies, that has prompted clinical studies to develop synergistic treatment protocols [1]. In 20-year follow-up studies of invasive breast cancer [2, 3], lumpectomy followed by radiotherapy reduced cumulative incidence of ipsilateral tumour recurrence from 39% to 14% [2]. With an increasing number of women opting for lumpectomy, ipsilateral tumour recurrence is becoming a significant clinical issue [4]. The NSABP B-06 and Milan trials demonstrated the therapeutic advantage of localised multi-

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modal therapy for breast cancer [2, 3]. Therefore, the application of localised chemotherapy to breast tissue may further improve clinical outcome.

Localised chemotherapy is already routine clinical practice for the treatment of high grade malignant glioma, where 1,3-bis-(p-carboxyphenoxy)propane and sebacic acid copolymer implants are used to deliver carmustine, commercially known as Gliadel Wafers [5, 6]. However, the development of local chemotherapy for breast cancer is still in pre-clinical development. To date, only a small number of studies have used synthetic hydrogels to deliver therapeutic payloads (e.g., chemotherapeutic agents, iron oxide nanoparticles, shRNA) using pH, temperature or proteolysis to trigger drug release [7–13]. No studies have focused on the design of films or wafers that can be applied directly to breast tumours. When developing such systems, it is crucial to use materials that are non-cytotoxic and biodegradable. A wealth of published biocompatibility studies have demonstrated that silk films with a wide spectrum of crystallinity are well tolerated *in vivo* with minimal inflammation or host immune response when implanted subcutaneously or directly into tissues (e.g. muscle, abdominal wall) [14–16]. Often the local tissue response is significantly better than seen for other clinically approved synthetic and degradable polymers (e.g. poly(lactic-co-glycolic acid) copolymers, polycaprolactone) or for collagen. Silk has been used for centuries in humans [17] and has recently also been approved for new medical applications, such as surgical meshes (Allergan Medical, Inc.). Overall silk protein, in particular, has an excellent clinical track record [18] and is easily processed into films [19]. We have previously shown with preliminary *in vitro* studies the potential of silk films to deliver proteins or low molecular weight compounds [20–22].

In the present study, silk films were prepared with doxorubicin, a clinically relevant chemotherapeutic agent [23]. This anthracycline is frequently used as a (neo)adjuvant chemotherapeutic agent for breast cancer [1, 23]. We hypothesised that the local delivery of doxorubicin would improve clinical outcomes in terms of tumour response. First, we investigated the interactions between silk and doxorubicin by generating silk films with different β -sheet contents and examined both adsorption (affinity) and release kinetics. Next, we tested the therapeutic potential of these doxorubicin-loaded silk films *in vitro*, and subsequently, in a humanised orthotopic breast cancer model to provide insight into local tumour response and metastasis.

MATERIALS AND METHODS

Silk film preparation and loading with doxorubicin for *in vitro* and *in vivo* studies

A *Bombyx mori* fibroin solution was prepared as we have described previously [24] and diluted in water to yield a 4% (wt/v) solution. Next, 4 ml of this solution was cast onto 4.5 × 4.5 cm polydimethylsiloxane (PDMS) (Dow Corning Corporation, Midland USA) substrates and allowed to dry at 23°C and 60% relative humidity. The resulting films were soluble in water. To generate insoluble silk films with varying amounts of crystallinity (beta-sheets), films underwent water vapour annealing at (1) 4°C for 12 h, (2) 25°C for 6 h, (3) 60°C for 12 h, or (4) 20 min steam autoclaving at 121°C. Following water vapour annealing, the samples were stored in a desiccator at room temperature. Further details regarding the water vapour annealing process have been described in detail elsewhere [25]. Water vapour annealed silk films were loaded with doxorubicin (LC Laboratories, Woburn USA) by soaking them in a doxorubicin solution and monitoring drug depletion from solution using UV-Vis absorption spectroscopy at 508 nm. To generate water soluble silk films with a nominal doxorubicin loading of 40 µg for each 12 mm film, the drug was dissolved in ddH₂O and 1.13 mg of drug was added to the silk solution prior to casting on PDMS (Fig. 1a, b). A stack of 10 dry films was used to determine film thickness. Next, films were

allowed to hydrate in PBS for 24 h and measured again with a micrometer to determine swelling.

Silk films: Uptake kinetics, equilibrium binding and release kinetics of doxorubicin

To determine the binding kinetics of doxorubicin to annealed silk films, the samples were incubated with 2 ml of doxorubicin (100 µg/ml) under dynamic conditions at room temperature. Doxorubicin depletion from solution was monitored at 508 nm by UV-Vis absorption spectroscopy. For equilibrium adsorption studies, silk films were incubated with varying amounts of doxorubicin (0.04 – 5 mg) for 48 h and analysed as detailed above. To determine adsorption constants (K_D), data were fitted using total binding by least square regression using GraphInStat 3.06 (GraphPad Software, LA Jolla, CA, USA). To monitor doxorubicin release from silk films, samples were incubated with 1 ml of PBS and cumulative drug release was monitored by measuring doxorubicin-associated fluorescence (excitation wavelength 485 nm, emission wavelength 590 nm) under sink conditions at room temperature.

In vitro degradation studies of silk films

Silk films were incubated with either PBS alone or with a Tris-CaCl₂ buffer (100 mM Tris-HCl, 50 mM CaCl₂, pH 7.8) containing 2 U of alpha-chymotrypsin per 15 mg of silk (Sigma-Aldrich, St Louis USA). The solutions were replaced every 3 days. After 3 weeks, the films were washed with ddH₂O and dried over night at 60°C. The mass loss was determined by weighing.

In vitro cytotoxicity studies

The adenocarcinoma breast cancer cell line MDA-MB-231 was obtained from ATTC (Manassas USA). The cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C and were routinely subcultured every 2–3 days. MDA-MB-231 cells were grown in RPMI 1640 supplemented with 10% (v/v) FBS. The *in vitro* toxicity of doxorubicin-loaded silk and diffusible doxorubicin samples were determined by plating 8×10^3 cells/cm² on Transwell inserts (0.4 µm pore size, Corning, New York USA) in 100 µl of medium with 500 µl of medium in the lower chamber. The cultures were allowed to recover for 24 h. Next, soluble silk films, stabilised silk films (autoclaved to induce beta-sheets) and the respective doxorubicin-loaded silk films containing 40 µg of the drug were added to the lower chamber. A 40 µg dose of freely diffusible doxorubicin was used as a control (i.e. no silk in the system). For endpoint studies, cell viability was determined after 72 h of exposure using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT at 5 mg/ml) as a substrate. Following a 5-h incubation period, formazan was solubilised with dimethylsulfoxide, and the absorbance was measured at 560 nm. For long-term assessments, analogous studies were performed using AlamaBlue (Invitrogen, Grand Island USA) instead of MTT. At the indicated time points, 50 µl of AlamaBlue (Invitrogen, Grand Island USA) was added to the culture medium, and cell viability was measured after a 4-h incubation period by monitoring fluorescence (excitation wavelength 550 nm, emission wavelength 590 nm). Next, the medium was replaced with fresh culture medium and cultures were continued. To mimic disease relapse, culture inserts were re-seeded at day 6 with 8×10^3 cells/cm² and cell viability was monitored as described above. The only two culture conditions that were not re-seeded were the control cultures and the soluble silk film cultures because they had become confluent by this time point.

In vivo tumour studies and autopsy

All *in vivo* studies were approved by the Institutional Animal Care and Use Committee (Protocol B2010-101), and the animals were maintained under the guidelines established by

the National Institute of Health and Tufts University. Breast tumours were induced by inoculating MDA-MB-231 tumour cells that metastasised following orthotopic injection in mice [26]. Cells were genetically modified to carry the firefly luciferase gene to allow *in vivo* bioluminescence imaging. Female NOD/SCID mice (NOD.CB17-Prkdc^{scid}/NcrCrI), 6 to 10 weeks in age, were purchased from Charles River (Wilmington, USA). For surgeries, the animals were anaesthetised using isoflurane, shaved and the surgical area was cleaned. A total of 5×10^5 cells in 20 μ l Matrigel (BD Biosciences, Bedford USA) were injected bilaterally into the 4th or 5th mammary fat pad using a Hamilton syringe equipped with a 22-Gauge needle. Twelve days post-tumour inoculation, mice received circular silk films (ϕ 12 mm) at the primary tumour sites during a second surgery that placed a film between the tumour-bearing mammary fat pad and the abdominal wall. The following treatment groups were studied: (1) soluble silk films loaded with 40 μ g/film doxorubicin, (2) 25°C water vapour annealed silk films (denoted as stabilised silk films) loaded with 40 μ g/film doxorubicin, (3) 25°C water vapour annealed silk films (denoted as stabilised silk films) that served as a control film. To provide a direct comparison for the doxorubicin silk film groups (2×40 μ g doxorubicin/mouse), the equivalent dose of doxorubicin (80 μ g) was administered in 100 μ l of PBS via tail vein injection to the respective control group (denoted as doxorubicin i.v.). Five animals were used for each treatment group. The only exception was the stabilised silk film control group, where four animals were used. Disease progression was monitored weekly with intraperitoneal injections of D-luciferin (Molecular Probes, Eugene USA) followed by measuring tumour cell-associated bioluminescence using the Xenogen IVIS 200 imaging system using the Living Image Software 4.2 (Caliper Life Sciences, Hopkinton USA). At the study endpoint, brain, lung, liver and bones were examined for metastasis by dissecting them from the carcass. Tibia and femur from hind legs were harvested and dissected free from muscle and tendons to serve as bone samples. Organs were imaged at maximum sensitivity to detect metastatic cancer cells. Tissues were scored for the presence or absence of metastasis. Primary tumours were dissected and weighed. The abdominal wall that was in direct contact with the silk film was retrieved, fixed in buffered formalin and processed for paraffin embedding. The samples were sectioned at the transverse plane at two levels and stained with Haematoxylin and Eosin (H&E). Hearts were also removed, weighed and processed for H&E staining as described above. A board-certified veterinarian pathologist examined the cardiac sections for signs of doxorubicin-associated toxicity using a published scoring system [27].

Statistical analysis

Data were analysed using GraphPad Instat 3.06 (GraphPad Software, La Jolla USA). Sample pairs were analysed using the Student's *t*-test. Multiple comparisons were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc tests to evaluate the significant differences (* *P* 0.05, ** *P* 0.001, *** *P* 0.0001) among the samples and controls. All error bars represent the standard deviations (SD) with the exception of the *in vivo* study where error bars represent the standard error of the mean (SEM).

RESULTS

Doxorubicin binding and release from silk films

To systematically study doxorubicin-silk interactions, a set of silk films with different crystallinity contents, or beta-sheets, was generated using water vapour annealing (Fig. 1a, b). Water vapour annealing at 4°C, 25°C, 60°C and 121°C induces beta-sheet contents accounting for approximately 14%, 30%, 50% and 57%, respectively, of secondary structure [25]. A crystallinity of 57% is the maximum amount that can be achieved using *Bombyx mori* fibroin. Untreated silk films have a beta-sheet content of 10% and are water soluble. The use of PDMS surfaces, controlled humidity and minimal air flow during film drying

helped to achieve uniform films that displayed minimal defects (e.g., blisters, ripples, and variable thickness). The average thickness of the films was 43 μm , which increased to 49 μm after swelling in PBS (Fig. 1c). Dry films were self-standing (Fig. 1d), a property that was retained following film hydration (Fig. 1e). Hydrated films also followed surface contours (Fig. 1e). Films annealed at 4°C and 25°C have similar mechanical properties with an elastic modulus of 20 MPa and a yield stress and tensile strength of 2 MPa [25]. Films annealed at 60°C and 121°C showed a similar trend to the 4°C, and 25°C annealed samples, though with mechanical properties approximately 3 fold higher [25]. Two methods were developed for doxorubicin loading. The first method relied on doping the silk solution with doxorubicin prior to casting. The second method used stabilised silk films that were soaked in a doxorubicin solution (Fig. 1a, b). The first method allowed the formation of drug-loaded silk films that dissolved rapidly in water (within 60 seconds) (Supplementary movie. 1). Drug loading could be readily controlled (Fig. 1f) by varying: (1) the amount of doxorubicin in the doping solution that was added to the silk solution prior to casting, or (2) the amount of doxorubicin in the aqueous phase and/or the exposure time during drug adsorption to stabilised silk films. The uptake kinetics for 200 μg of doxorubicin into stabilised silk films was rapid and reached > 90% drug loading after 60 min in the 25°C water vapour annealed silk films (Fig. 2a). Similar kinetic behaviour was observed for films that were exposed to 4°C, 60°C and 121°C water vapour annealing (data not shown). The adsorption process of doxorubicin to stabilised silk films was further characterised by equilibrium adsorption studies; Fig. 2b depicts the typical binding isotherm of doxorubicin to 4°C water annealed silk films. Adsorption affinities increased with increasing crystallinity, specifically, $1.96 (\pm 0.30)$, $2.15 (\pm 0.05)$, $1.55 (\pm 1.00)$ and $0.70 (\pm 0.37)$ for samples that were water vapour annealed at 4°C, 25°C, 60°C and 121°C, respectively.

Next, doxorubicin release from films loaded with 40 μg of drug was studied over 4 weeks. Release profiles for all samples were similar and showed a more rapid release during the first 10 days that accounted for 70% of the cumulative drug release for all samples (Fig. 2c). However, the total amount of drug released from each film was significantly different and dependent on film crystallinity (Fig. 2c). The cumulative drug release from 4°C water vapour annealed films was 53% and decreased for 25°C (48%), 60°C (44%) and 121°C (39%) water vapour annealed films. To complement these studies, film stability was also examined. Films incubated in PBS for 4 weeks did not show significant degradation (data not shown). However, incubation with the serine protease α -chymotrypsin induced significant degradation (Fig. 2d) that contributed to 29%, 20%, and 11% mass loss for 4°C, 25°C, and 60°C water vapour annealed silk films, respectively. Silk films that were water vapour annealed at 121°C showed no mass loss.

In vitro cytotoxicity of doxorubicin loaded silk films

The cytotoxicity of doxorubicin-loaded silk films was tested using adenocarcinoma MDA-MB-231 breast cancer cells following a 3-day exposure (Fig. 3a). In this short-term cytotoxicity assay, stabilised silk films and soluble silk films were loaded with 40 μg of doxorubicin, and the cell response was compared with freely diffusible drug at the equivalent dose. Cell viability was significantly reduced to only 22% following exposure to freely diffusible doxorubicin. Both soluble and stabilised silk films loaded with doxorubicin also showed a significant reduction in cell viability, 18% and 27%, respectively. However, exposure to only soluble and stabilised silk films had no significant impact on cell viability (Fig. 3a). In addition to the 3-day cytotoxicity assay, a 12-day long-term assay was performed. To better mimic the *in vivo* pharmacokinetics of intravenous doxorubicin, culture medium was replaced throughout the 12-day culture period. Furthermore, cultures were re-seeded at day 6 with MDA-MB-231 breast cancer cells to mimic disease relapse. Control cultures and cultures exposed to soluble silk films demonstrated exponential cell

growth during days 3 through 6 and less growth during the remaining days in culture. The doxorubicin-treated groups showed little growth during the first 8 days in culture. However, soluble silk films with doxorubicin and diffusible doxorubicin treatment groups both showed significant cell growth at day 8 compared with stabilised silk films loaded with doxorubicin. This difference became more pronounced at day 12. The ability of doxorubicin-loaded silk films to inhibit cell growth over extended periods of time *in vitro* prompted the *in vivo* studies.

In vivo anti-tumour studies of doxorubicin-loaded silk films

The therapeutic impact of delivering the equivalent amount of doxorubicin via silk films or via a bolus intravenous injection was studied using an aggressive adenocarcinoma cell line (Fig. 4). Orthotopic tumours were inoculated, and therapeutic intervention was initiated once tumours were established. Stabilised silk films containing doxorubicin demonstrated a significant primary tumour response from week 3 onwards, as determined by cancer cell-specific bioluminescence imaging (Fig. 4a). This tumour response was significantly greater than was observed in mice that had received an equivalent amount of intravenous doxorubicin (Fig. 4a). There was no significant difference between mice that had received soluble silk films with doxorubicin or stabilised silk films without drug. At week 5, the best tumour response at the orthotopic site was observed in mice receiving stabilised silk films that contained doxorubicin, followed by soluble silk films with doxorubicin (Fig. 4b). A similar trend was seen at week 6 (Fig. 4c). A reduced tumour burden was observed in mice that received intravenous doxorubicin. There was no significant difference between control mice and animals that had been treated with silk films without drug (Fig. 4b–d). Animals were euthanised at week 6, and primary tumours were removed and weighed (Fig. 4d). The average tumour weight for the control group was comparable to animals that had only been treated with stabilised silk films. The greatest reduction in tumour burden was observed in animals that received stabilised silk films loaded with doxorubicin, followed by soluble silk films loaded with doxorubicin and intravenous doxorubicin (Fig. 4d). There was a good correlation between the tumour weights and the cancer cell-associated bioluminescence signal detected by non-invasive imaging (Fig. 4e). In addition to examining primary tumour growth, we also determined metastatic spread into bones, lung, liver and brain (Fig. 4f). All untreated control mice had bone metastasis and a high prevalence for lung and liver metastasis (60%), followed by metastatic spread to the brain (40%). A similar metastatic spread was observed for mice that only received stabilised silk films. However, animals that received intravenous doxorubicin had a reduced prevalence of bone (80%) and liver (40%) metastasis, and no brain metastases were observed. This clinical outcome was further improved for animals that received soluble silk films that contained doxorubicin; lung and liver metastasis was only observed in 20% of the animals. Animals treated with stabilised silk films that contained doxorubicin showed no metastasis to the brain and liver, and only low levels of metastatic spread to the lungs (20%) (Fig. 4f).

Autopsy to determine doxorubicin-associated toxicity

The heart weight was used to examine systemic doxorubicin-associated toxicity [28]. No significant difference was observed between control animals and mice that received stabilised silk films with or without doxorubicin (Fig. 5). However, there was a significant reduction in heart weight for animals that received either a bolus intravenous injection of doxorubicin or the equivalent dose via soluble silk films. To further examine cardiotoxicity, H&E staining of hearts was performed and analysed. All treatment groups showed similar cellular morphology and H&E staining (Supplementary Fig. 1). There were no signs of myocardial remodelling or scarring in any of the animals that either received doxorubicin or served as controls. Doxorubicin is a known vesicant [23], and therefore, the abdominal wall that was exposed to doxorubicin-loaded silk film was also subjected to histological

examination (Fig. 5a, c). Stabilised silk films with no drug showed neither macroscopic nor microscopic changes. Soluble silk films that contained doxorubicin showed some morphological changes at the microscopic level that were characterised by the deposition of collagen and cellular infiltration. However, stabilised silk films that contained doxorubicin did not show these signs and were comparable to the controls.

DISCUSSION

In the United States alone, more than 230,000 new breast cancer cases are diagnosed annually. The vast majority of breast cancer-related deaths are due to blood-borne metastasis, [29] and thus, localised chemotherapy may seem to be the wrong approach to treat breast cancer. However, clinical trials have determined that high-risk pre-menopausal women benefit from local radiotherapy by reducing ipsilateral tumour recurrence, resulting in increased disease-free and overall survival [30], which demonstrated that locoregional control is required to achieve prolonged survival. Considering the clinical benefit of systemic chemotherapy for local or regional control following mastectomy is low [31], localised chemotherapy to the primary tumour site may further improve clinical outcomes. An additional indication for the use of localised chemotherapy may be the treatment of locally advanced breast cancer. For these patients, the results of mastectomy have been disappointing, and surgery is only indicated for debulking operations that can prove valuable when combined with radiotherapy, chemotherapy and hormonal therapy [1].

Therefore, we studied the ability of silk films to serve as a delivery platform for the clinically relevant chemotherapeutic agent doxorubicin. By adjusting the crystallinity of the silk films, we were able to manipulate the adsorption affinity. The affinities were in the low micromolar range, and thus, on the order of low-fidelity receptor-ligand interactions [32]. To our knowledge, this is the highest affinity that has been reported for doxorubicin using a polymeric delivery vehicle. This strong affinity likely results from adsorption of the hydrophobic doxorubicin to the hydrophobic core repeats of the silk. By increasing the β -sheet content, these hydrophobic domains provide more adsorption sites [33]. In addition to providing hydrophobic adsorption sites, the negative net charge of silk is also expected to contribute to drug binding, analogous to that observed in doxorubicin-heparin complexes [34]. The desorption of doxorubicin from silk was greatest in the 4°C water annealed silk films, which further supports the idea that β -sheet content regulates doxorubicin-silk interactions and kinetics. The absence of silk degradation during the release study excluded the possibility of erosion-dependent drug release.

A number of different silk film preparations were studied *in vitro* demonstrating that neither soluble nor stabilised silk films affected cell viability in either short- or long-term cultures. This observation is in agreement with the general notion that silk is well tolerated and has minimal impact on cells and tissues [18]. By manipulating drug release from the silk films, we showed that stabilised silk films loaded with doxorubicin inhibited cell growth in long term *in vitro* studies. Next, it was important to examine the therapeutic potential *in vivo*; previous studies examining the potential of local tumour therapy have mostly used microparticles in non-breast tumours [13]. A few studies have used gels [8, 10, 12, 35–38] or polymer drug conjugates [39–41], and no studies have been reported for films. For the first time, we have used a humanised orthotopic breast cancer model that allowed us to examine both locoregional control, and its impact on metastasis following local therapy. Over the time course of the study, stabilised silk films loaded with doxorubicin outperformed all other treatments, including animals that received the equivalent dose of doxorubicin via an i.v. bolus injection. The excellent locoregional response following exposure to stabilised silk films with doxorubicin was reflected in a reduction in tumour weight, decreased cancer cell-specific bioluminescence and reduced metastatic spread. This

study also demonstrated that even soluble silk films loaded with doxorubicin provided better disease control than systemic chemotherapy. This model seems to recapitulate some clinical traits, where systemic chemotherapy only provides marginal control over local or regional disease [31]. Animals that received stabilised silk film controls showed no statistically significant tumour response, indicating that the surgical intervention required for film insertion had no impact on tumour growth. Furthermore, the presence of the silk film did not alter disease progression by any mechanical mechanism (e.g., barrier function to tumour growth). Taken together the delivery of doxorubicin via stabilised silk films is well suited to achieve locoregional control over tumour growth, and an overall reduction in metastatic burden. It is likely that the locoregional control of the disease contributed to reduced metastasis, as likely fewer cancer cells were able to escape from the primary tumor to seed distant sites. Conversely, poor local control is known to contribute to disease progression and metastasis due to the shedding of large numbers of tumor cells into the circulation which eventually establish secondary tumors (i.e. metastasis) [29]. However, only recently has the response of the tumor microenvironment to chemotherapy been studied [42] and it remains to be established to what extent doxorubicin also impacts premetastatic sites *per se* (e.g. brain, lung, liver, bone). A potential limitation of the present study is the absence of partial tumour resection prior to therapy; such strategies might be employed in an attempt to mimic patient care. However, we decided not to perform partial tumour resection in mice, because (1) makes it easier to observe a good therapeutic response and thus skews the data set in favour of focal therapy, (2) introduces additional variables that are difficult to control for [43].

In the clinic, systemic side effects associated with doxorubicin limits its use to a maximum cumulative dose of $\sim 300 \text{ mg/m}^2$; although myelosuppression is a serious side effect associated with the drug, cardiotoxicity is a major concern for patients treated with doxorubicin, as it leads to congestive heart failure [44]. In addition to limiting the total cumulative exposure to $\sim 300 \text{ mg/m}^2$, metronomic infusions or liposomal doxorubicin formulations are used to avoid high plasma levels of the drug. By changing doxorubicin delivery from a systemic application to localised therapy, one may also reduce cardiotoxicity. Previous studies have demonstrated that heart weight can serve as an indicator of doxorubicin-associated cardiotoxicity [28]. The systemic delivery of doxorubicin and its delivery via soluble silk films resulted in a significant reduction in heart weight, which is suggestive of cardiotoxicity. In contrast, stabilised silk films with doxorubicin showed no significant difference to controls. *In vivo* studies in mice examining doxorubicin-associated cardiotoxicity have frequently used intraperitoneal injections [28]. Therefore, the rapid release of doxorubicin from soluble silk films at their implantation sight is also likely to induce systemic toxicities. However, histological examination of the cardiac tissue did not show consistent morphological changes associated with doxorubicin-induced toxicity, as reported in previous studies [28, 45, 46]. The 2 – 4-fold lower cumulative dose used in the present study could potentially account for this discrepancy. Histological examination of the abdominal wall exposed to soluble silk films loaded with doxorubicin showed a local tissue response, suggesting that the rapid release of doxorubicin was paired with both systemic and local adverse effects. However, stabilised silk films loaded with doxorubicin did not show systemic or local adverse tissue responses. The biocompatibility of silk, and its ability to slowly degrade *in vivo* over 6–10 months [18], makes silk an excellent drug carrier. In addition to the demonstrated therapeutic benefits of local doxorubicin delivery, the inherent ability of silk to slowly release doxorubicin is a unique feature to this biomaterial. Collectively, this bodes well for the future use of this therapeutic platform for localised chemotherapy.

CONCLUSIONS

In summary, the manipulation of silk crystallinity provides control over doxorubicin release, which minimises systemic and local adverse effects but maximises therapeutic impact. This proof of concept study demonstrated that silk films loaded with doxorubicin provide locoregional control of human breast cancer *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Preparation of doxorubicin-loaded silk films. **(A)** Strategies to generate doxorubicin-loaded films that are water-soluble or **(B)** water-insoluble with variable β -sheet content. **(C)** Film thickness of dry and hydrated silk films ($\pm$ SD; $n = 8$). **(D)** Free-standing doxorubicin-loaded silk films in the dry state (scale bar 7 mm). **(E)** Free-standing hydrated films following surface contours (scale bar 7 mm). **(F)** Silk films can be loaded with a range of doxorubicin concentrations.

Figure 2.

Doxorubicin interactions and film stability. **(A)** Typical adsorption kinetics of doxorubicin to silk monitored by following doxorubicin depletion from solution using UV-Vis spectroscopy. **(B)** Equilibrium binding studies of doxorubicin to silk films. Graph depicts data for 4°C water vapour annealed silk films. **(C)** Cumulative release of doxorubicin into PBS. Significant differences between 4°C water vapour-annealed silk films and other groups was determined by ANOVA followed by Dunnett's multiple comparison post hoc test, * $P < 0.05$; $\pm$ SD; $n = 4$. **(D)** *In vitro* degradation of silk films with α -chymotrypsin. Significant differences between 121°C water vapour annealed silk films and other groups was determined by ANOVA followed by Dunnett's multiple comparison post hoc test, *** $P < 0.0001$; $\pm$ SD; $n = 4$).

Figure 3.

In vitro toxicity of doxorubicin-loaded silk films towards human breast cancer cells. **(A)** MDA-MB-231 cell viability following a 72-h exposure to soluble or stabilised silk films $\pm$ doxorubicin and freely diffusible doxorubicin at the equivalent dose. Significant differences between control cells under optimum growth conditions (plasma-treated polystyrene, PTP) and other groups were determined by ANOVA followed by Dunnett's multiple comparison post hoc test, ns = not significant, *** $P < 0.0001$; $\pm$ SD; $n = 3$). **(B)** Long-term toxicity of film preparations and diffusible doxorubicin. Culture medium was replaced at the indicated time point. With the exception of control wells or wells containing stabilised silk, wells were reseeded with MDA-MB-231 cells at day 6. Significant differences between diffusible doxorubicin and the other doxorubicin treatment groups was determined by ANOVA followed by Dunnett's multiple comparison post hoc test, ns = not significant, *** $P < 0.0001$; $\pm$ SD; $n = 4$).

Figure 4.

In vivo response of orthotopic breast tumours to doxorubicin-loaded silk films. (A) Cancer cell-associated bioluminescence at week 3, (B) 5 and (C) 6 weeks. (D) Weight of primary tumours at the end of the study (week 6). (E) *In vivo* tumour cell-specific bioluminescence of representative mice from each treatment group at week 6. Plot symbols are defined in panel (C). (F) Metastatic spread of cancer cells to organs at week 6. Plot symbols are defined in panel (C). Significant differences between control animals and the other treatment groups was determined by ANOVA followed by Dunnett's multiple comparison post hoc test, ns = not significant, **P < 0.001; ± SEM; n = 5 except for stabilised silk films where n = 4).

Figure 5.

In vivo toxicity following systemic and local doxorubicin application. **(A)** Image depicting tumour and stabilised silk film at the time of necropsy (week 6, scale bar 5 mm). **(B)** Heart weights of animals treated with localised or systemic doxorubicin and the respective controls. Significant differences between control animals and the other treatment groups was determined by ANOVA followed by Dunnett's multiple comparison post hoc test, ns=not significant, ** $P < 0.001$, *** $P < 0.0001$; $\pm$ SEM; $n = 5$ except for stabilised silk films where $n = 4$. **(C)** Histological section and H&E staining of the abdominal wall exposed to stabilised or soluble silk film $\pm$ doxorubicin. (Scale bar 200 μ m).