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Incorporation of Proteinase Inhibitors into Silk-Based Delivery Devices for Enhanced Control of Degradation and Drug Release

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Abstract

Controlling the rate of silk degradation is critical to its potential use in biomedical applications, including drug delivery and tissue engineering. The effect of protease concentration on accelerating degradation, and the use of ethylenediamine tetraacetic acid (EDTA) on reducing rates of degradation and on drug release from silk-based drug carriers was studied. Increased rates of proteolysis resulted in increased dye release from silk carriers, while EDTA release from the silk carriers inhibited proteolysis. The sustained release of EDTA from silk carriers in combination with the release of the small molecule anticonvulsant adenosine was investigated *in vitro*. This combination of factors resulted in delayed release of adenosine by inhibiting proteolytic activity. These results introduce a promising strategy to control drug delivery through the regulation of silk degradation rate, achieved via manipulation of local proteolytic activity. This ability to modulate enzyme function could be applicable to a range of silk biomaterial formats as well as other biodegradable polymers where enzymatic functions control biomaterial degradation and drug release rates.

1. Introduction

The systemic periodic administration of drugs, such as via oral delivery or repeated injections, has been the primary mode of drug delivery for decades. However, this approach suffers from many disadvantages. Systemically administered drugs must be distributed throughout the entire body and large doses are usually required to maintain sufficient drug concentration at the target site, resulting in unwanted side effects [1]. In addition, this delivery approach results in peaks and valleys in drug levels at the target delivery site rather than maintaining constant local drug levels within the desired therapeutic range [2]. Issues of patient compliance [3], convenience [4] and cost [5] also suggest that alternatives to systemic periodic administration of drugs would be attractive.

To address these disadvantages, targeted, site-specific, controlled release of drugs via implantable polymer carriers has been proposed. Synthetic, non-degradable materials have been primarily studied to meet this need, while biodegradable implants offer the advantage

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of alleviating the need for surgical removal of the materials after the conclusion of therapy [4]. A variety of biodegradable polymers have been studied for this application, but the ability to achieve sustained, zero-order release, of small molecules from implantable fully degradable implants remains a major unmet challenge. This challenge emerges in part from the inability to generate highly crystalline biomaterials with tight control of processing windows as regulators of diffusion rates, without damage to the entrapped drug.

Silk is a biologically derived protein polymer that meets the necessary biomaterial requirements for implantable and degradable medical applications. Silk exhibits biocompatibility [6–8], is mechanically strong [9], and degrades to non-toxic products *in vivo* [10–11]. Silk can be processed into a wide range of material formats including vehicles for drug delivery [12] and highly porous 3D scaffolds for tissue engineering [13,6]. Silk is particularly attractive for sustained drug release applications because the degradation time frame can be controlled from weeks to years via regulation of beta sheet (crystallinity) content during processing [10,11]. Due to this unique combination of properties, a range of different silk material formats have been investigated as controlled release drug carriers, including bulk loaded systems [15], microspheres and nanoparticles [16–20], nanofilms [21] and encapsulated reservoirs [22]. An extensive review on delivery of bioactive compounds from silk carriers has also been published [12].

Controlled loading and delivery of the small molecule anti-convulsant adenosine from silk carriers has been demonstrated in a series of *in vitro* and *in vivo* studies. In studies of silk encapsulated solid reservoirs of adenosine, a linear release profile was observed for 14 days and the release rate was dependent on the thickness of the silk encapsulating coating [22]. The therapeutic potential of adenosine-releasing silk drug delivery systems was validated in a rat model of kindling epileptogenesis [23,24]. Adenosine-releasing silk microspheres were embedded in porous silk sponge rods which were then coated with additional burst-delaying silk capping layers and these were implanted into the hippocampi of rats prior to the onset of epilepsy development. Robust seizure suppression was seen in animals receiving silk implants designed to release approx. 1 μ g of adenosine per day compared to animals receiving empty (control) silk implants [24]. This effective dose at a specified target site would not be feasible by systemic application of adenosine given a high rate of systemic clearance, poor penetrability of the blood-brain barrier and cardiovascular side effects of systemic adenosine delivery. These *in vivo* studies demonstrated the therapeutic benefits of localized and sustained release of small molecule drugs from silk systems.

Silk degradation has been studied in depth while the effect of the degradation process on drug release has not been characterized. The biodegradation of other protein-based delivery systems, such as collagen and gelatin have been reported to be related to drug release rate [25–26]. Degradable drug carriers including silk, poly(lactic-co-glycolic acid) (PLGA), collagen, gelatin and hyaluronic acid (HA) release drugs dependent on diffusion, degradation or a combination of both release mechanisms. The contribution that degradation plays in the overall release profiles depends on the diffusivity of the drug through the polymer carrier as well as the rate of degradation of the carrier. For low molecular weight drugs free diffusion results in rapid release while higher molecular weight molecules are less able to diffuse and rely more on enzymatic degradation or chemical hydrolysis to liberate the entrapped drug [27]. Collagen degradation (often simulated *in vitro* by incubation with bacterial collagenase, cathepsin, pepsin or trypsin [25]) impacts drug release. More rapid release of fluorescein isothiocyanate (FITC) from crosslinked collagen hydrogels incubated with *Clostridium histolyticum* collagenase type I was found when compared to PBS alone [1]. The importance of accounting for the effect of the therapeutic release on cellular responses in designing drug releasing collagen systems has also been stressed [25]. For example, steroids modify the immune response, potentially slowing degradation [28] and

growth factors or immunostimulants can impact cellular responses, potentially altering degradation rates [29]. In addition, disease states can be associated with increased or decreased proteolytic activity at implantation sites. For example, increased matrix metalloproteinase activity in and around chondrocytes in osteoarthritic cartilage [30], increased extracellular matrix (ECM) degrading proteinase activity in glioma tissue [31], increased alpha-chymotrypsin inhibitor activity in the cerebrospinal fluid of patients with Alzheimer's disease [32] have been reported. Young *et al.* [26] reported that crosslinking density correlated with the rate of gelatin carrier degradation, which in turn controlled the release of growth factors from gelatin hydrogels. These data suggest that release rate from gelatin hydrogels can be controlled by manipulation of the rate of carrier degradation. Kurisawa *et al.* [33] found that increasing hyaluronidase concentration increased degradation rate. Luo *et al.* [27] found that FITC loaded hyaluronic acid (HA) hydrogels incubated with hyaluronidase exhibited increased release rates compared to controls without enzyme.

In addition to natural degradable materials, there are numerous examples in the literature of designer polymers linked to drugs with protease-specific peptide spacers, such that drug release is mediated by protease activity. Examples include biomaterials for chemotherapeutic delivery where the linker between the polymer carrier and the drug is designed to be cleaved intracellularly by lysosomal enzymes [34]. Other examples include polymer delivery systems with drug-linkers designed to be specifically cleaved by matrix metalloproteinases overexpressed by tumors [35] or urokinase plasminogen activator (uPA), a proteinase involved in cancer progression [36–37]. These systems exhibit combine degradation and drug release that proceeds via dose-dependent enzymatic cleavage.

Poly(lactic-co-glycolic acid) (PLGA) systems which degrade via chemical hydrolysis rather than enzymes also exhibit increased rates of drug release with increased degradation rate. The degradation of PLGA can be manipulated by buffer pH, [38] temperature [39] and monomer ratio [40–43]. These studies demonstrate that increased polymer carrier degradation rate can increase drug release. In temperature-induced degradation of the PLGA drug carrier, both release and degradation rate were almost ten times faster at 60°C versus 37°C [39].

Previously, we demonstrated that solid adenosine reservoirs encapsulated with silk exhibit more rapid release in a 0.1 mg/mL protease type XIV solution than in phosphate buffered saline (PBS) alone. As a result of the increase in release rate, the duration of release nearly doubled in the absence of proteinase (the time at which 50% of the total drug load was released, $t_{50} = 3.1$ days in proteinase compared to $t_{50} = 6.0$ days in PBS [22]. These results suggest that proteolysis of the silk drug carrier can impact drug release, which may provide an important potential control point for customizing silk based drug delivery systems. In addition, confirmation that proteolysis affects drug release from silk is important in developing *in vitro* models for testing silk delivery systems that better reflect local, implantation-site-specific proteinase activity.

Therefore, the objective of the present study was to investigate the effect of proteinase concentration on drug release from silk carriers, as well as the effect of an enzyme inhibitor release on the proteinase/silk degradation/drug release process. The goal was to determine if inhibition of protease type XIV activity via the co-release of a small molecule inhibitor could be used as a further control point in terms of control of release of a therapeutic drug from silk. While protease concentration is important in control of drug release from silk carriers, inhibition-based control of drug release may provide both additional levels of control of the process, as well as control *in vivo*, since proteinase concentrations at the target

site of carrier implantation are fixed while the loading and release of inhibitors can be modulated in the device design.

2. Materials and Methods

2.1. Materials

Cocoons of *Bombyx mori* silkworm silk were purchased from Tajima Shoji Co., LTD (Sumiyoshicho, Naka-ku, Yokohama, Japan). Phosphate buffered saline (PBS) supplemented with 100 mg/L $MgCl_2$ and 100 mg/L $CaCl_2$ was purchased from Invitrogen (Carlsbad, CA). Protease type XIV (3.5 units/mg; mixture of at least three caseinolytic activities and one aminopeptidase) from *Streptomyces griseus* and all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Silk fibroin solution preparation

Silk fibroin was prepared from the cocoons as we have previously described [6]. Briefly, cocoons were boiled for 60 minutes in a solution of 0.02 M Na_2CO_3 and rinsed, then dried at ambient conditions overnight. The dried fibroin was solubilized in a 9.3 M aqueous LiBr solution at 60°C for 4 hours, yielding a 20% (w/v) solution. LiBr was then removed from the silk by dialyzing the solution against distilled water for 2.5 days using Slide-a-Lyzer dialysis cassettes (MWCO 3,500, Pierce Thermo Scientific Inc., Rockford, IL). Silk fibroin concentration was determined by evaporating water from a solution sample of known volume and massing using an analytical balance. The silk solution was concentrated by osmotic stress as previously described [13]. Briefly, silk fibroin aqueous solution (8% (w/v), 10 mL) was dialyzed against a 10–25% w/v PEG (10,000 g/mol) solution at ambient conditions, causing water molecules to move from the silk fibroin solution into the PEG solution through the dialysis membrane. All solutions were stored at 4–7°C before use.

2.3. Silk film preparation

Drug loaded silk fibroin films were prepared as we have previously described [15]. Briefly drug solution was mixed with silk to a final concentration of 8% (w/v) silk and aliquoted into teflon coated molds, then dried overnight at ambient conditions and removed from the molds after 24 hours, and treated with methanol for 5 minutes to increase beta sheet content to render the films water insoluble.

2.4 Adenosine powder reservoir preparation and encapsulation

Press-fit adenosine reservoirs were prepared and coated as we have previously described [22]. Briefly, approx. 100 mg of powder (50%/50% EDTA/adenosine, 50%/50% mannitol/adenosine or 100% mannitol) were loaded into the chamber of an Econopress (Sigma-Aldrich, St. Louis, MO) and pressed into solid tablets according to manufacturer instructions. Finished solid powder reservoirs selected for study were all within ± 5 mg of 70 mg and finished reservoir dimensions were 10 mm in diameter by 2 mm in height. Mannitol, a commonly used chemically inert filler material for compressed tablets [44], was used as a control. Reservoirs were coated by dipping in a 12% (w/v) aqueous silk solution, drying for 30 min at 60°C, then immersing in methanol for 5 minutes to increase β sheet content for aqueous-insolubility.

2.5 Reactive Red 120 and Indigo Carmine Films Release Studies in Phosphate Buffered Saline (PBS) and Proteinase Type XIV

For degradation studies, the dyes reactive red 120 and indigo carmine were used (Figure 1). For varied concentration proteinase release studies, films were prepared from 8% (w/v) silk containing either 1 mg of reactive red 120 or indigo carmine per film and 24 mg of silk (0.3

mL of 8% (w/v) silk per film). For the co-release studies, the drug was adenosine (Figure 1) and the proteinase inhibitor was ethylenediamine tetraacetic acid (EDTA) (Figure 1).

To determine dye release from encapsulated reservoirs, the silk films were immersed in 1 mL of Dulbecco's PBS at 37°C or a 0.025 mg/mL, 0.05 mg/mL, or 0.5 mg/mL protease type XIV solution. At desired time points, the buffer was removed and replaced with fresh buffer. The amount of released dye was determined using UV-Vis spectroscopy at 535 nm and 610 nm for reactive red 120 and indigo carmine, respectively, and compared to standard curves. The amount of released compound in each sample was summed with the amounts at each previous time point and divided by the total amount to obtain cumulative release values. Four samples were tested per release buffer type (n=4) and each sample was assayed in triplicate.

2.6 Degradation/Mass Loss Film Studies

To determine the effect of EDTA release from silk films on film degradation, the films were prepared from 8% (w/v) silk containing 1 mg of EDTA, 0.5 mg of adenosine or 1 mg of EDTA and 0.5 mg of adenosine. Prior to study the mass of the films was determined. Films were then immersed in 1 mL of PBS or 1 mL of 0.1 mg/mL protease type XIV for 5 days. At desired time points, a subset (n=3) of the films was removed, dried overnight at ambient conditions and reweighed. The fraction of remaining mass was determined by dividing the mass at each time point by the initial mass value.

2.7 Encapsulated Reservoir Release studies in phosphate buffered saline (PBS) and Protease Type XIV

To determine adenosine and EDTA release from silk coated powder reservoirs, the samples were immersed in 1.5 mL of Dulbecco's PBS at 37°C or a 0.1 mg/mL protease type XIV solution (intended to mimic *in vivo* proteolytic degradation of the silk coatings [10,45]). At selected time points the buffer was removed and replaced with fresh buffer to approximate sink conditions (the immediate clearance of adenosine released from the implant into the brain parenchyma [46]). Fresh protease type XIV solution was prepared for each replacement. Three samples were tested per drug loading type (EDTA + adenosine, mannitol + adenosine and mannitol alone) and each sample was assayed in triplicate for each time point. The buffer that was removed periodically from the system was assayed for adenosine content using a modified fluorescence assay as previously described [23] and by measuring absorbance at 260 nm using a UV spectrophotometer.

The buffer was also assayed for EDTA content [47]. Briefly, 20 μ L of 1.3 mM nickel ammonium sulfate solution was added to 100 μ L of sample or EDTA standard solution of known concentration. Then 50 μ L of 1 mg/mL xylenol orange solution was added, the reaction was mixed and allowed to react 5 minutes and then the sample absorbance at 595 nm was determined with a UV spectrophotometer plate reader. The amount of released compound in each sample was summed with the amounts at each previous time point and divided by the half the total initial reservoir mass to obtain cumulative percent release.

2.8 Proteinase activity determination

The buffer collected from the adenosine/EDTA release studies was assayed for proteolytic activity using the Amplite™ Universal Fluorimetric red fluorescence protease activity assay kit (ABD Bioquest, Sunnyvale, CA) according to the manufacturer's instructions. Briefly, a fluorescent-casein substrate was added to samples of unknown activity and incubated 1 hour at room temperature. Following incubation, fluorescence was read at excitation/emission = 540/590 nm. Each sample was assayed in triplicate. The final fluorescence value was compared to a standard curve generated for protease type XIV. The amount of active

protease type XIV in each sample was divided by the amount of active protease type XIV added to the sample every 24 hours (0.1 mg/mL) to determine what fraction of proteinase activity retained during each 24 hour incubation with EDTA-releasing or non-EDTA-releasing silk encapsulated reservoirs.

2.9 Release kinetics

The release profiles were characterized by linear regression analysis by determination of regression coefficients, R^2 , as zero order release is expected to correspond to constant drug release with respect to time. Values of t_{50} were taken as the time at which 50% of the drug was released from the reservoir. The data for release of drug from encapsulated reservoirs was fit using Eq. (1) in order to assess possible release mechanisms. M_t/M_∞ corresponds to the percent of adenosine released at time t relative to the total amount of drug in the tablet; K is a release constant and n is the release exponent indicating the type of drug release mechanism [48].

$$M_t/M_\infty = Kt^n \quad [\text{eq.1}]$$

A fit to this equation is used to assess the relative importance of Fickian and zero order mechanisms in drug diffusion. Fickian transport is dominated by diffusion. Case II transport is dominated by polymer swelling and exhibits zero-order time independent release kinetics. When $n=0.5$, pure diffusion for drug release is present, when $n=1$ swelling-controlled drug release or Case II transport is indicated. Other values for n suggest anomalous transport kinetics from combined mechanisms of pure diffusion and Case II transport. For linear fit, Eq. 1 is modified to Eq. 2. In this case, n is obtained from the slope of the plot of $\log(\text{released}\%)$ versus $\log t$.

$$\log(\text{released}\%) = \log(M_t/M_\infty) = \log K + n \log t \quad [\text{eq.2}]$$

3. Results

3.1 Effect of rate of proteolytic degradation on drug release from silk films

The release of reactive-red 120 and indigo carmine from silk films was characterized in buffer containing varied amounts of protease type XIV to confirm that increasing the degradation of silk films would result in increased drug release. Cumulative release profiles for the two dyes are shown in Figure 2A and Figure 3A, respectively. Zero order release was observed for reactive-red 120 films over the first four days, likely due to the relatively high molecular weight and bulky structure of the dye. Correlation coefficients (R^2) were ≥ 0.93 for all release profiles ($R^2 = 0.99, 0.95, 0.93$ and 0.97 for $0, 0.025, 0.05$ and 0.1 mg/mL, respectively). Therefore, daily release rates were relatively constant. The average daily release rate of reactive-red 120 from silk films in % total loaded drug per day was 7.62, 11.08, 13.89 and 21.42 for release buffer containing $0, 0.025, 0.05$ and 0.1 mg/mL of protease type XIV, respectively. Figure 2B shows average daily release rate versus protease type XIV concentration in the release buffer (PBS alone represents 0 mg/mL protease type XIV). The average release rate of reactive-red 120 increased proportionally with increased proteolytic degradation.

Unlike the release profiles observed for reactive-red 120, the release of indigo carmine was non zero-order; burst release was observed on day 1 for all release buffers/protease type XIV concentrations, followed by decreasing daily release rates relative to time (Figure 3A). This is likely because indigo carmine is low molecule weight and does not chemically interact with the silk. The non zero-order release profiles and the 90%-100% total drug load

released within 4 days regardless of proteinase concentration suggest that the average daily release rate was not an appropriate way to represent the release profile to assess the effects of increasing proteolysis on indigo carmine release. Therefore, the release data were fit to the modified Siepmann Peppas equation (equation 2) and values of the Siepmann Peppas release coefficient, n , were used to empirically compare linearity of the release profiles. The graph of fitted n values versus proteinase concentration is shown in Figure 3B. The n values decreased with increasing proteinase concentration in the release buffer, suggesting that in a more diffusion-driven system the increased proteolysis decreases the linearity of drug release. The increasing linearity of the release curve suggests that since average release rates are increasingly constant, less total drug is released early in the process compared to a less linear (i.e. lower n) release curve.

Increasing proteolysis results in a decrease in release coefficient n (Figure 3B), which shows that increasing proteolysis increases release of indigo carmine from the silk films. For both dyes and release profiles tested (reactive red 120/zero-order release and indigo carmine/diffusion-driven release), increasing the protease type XIV concentration and thus increasing proteolytic degradation of the silk film drug carrier, results in an increase in drug release.

3.2 Effect of EDTA release on mass loss of silk films in protease type XIV

To investigate the effect of EDTA release from silk films on film degradation, mass loss was characterized for silk films loaded with drug (adenosine alone), loaded with nothing (control), loaded with inhibitor (EDTA alone) and co-loaded with both drug and inhibitor (EDTA+adenosine). Silk films were immersed in 0.1 mg/mL proteinase type XIV or in PBS (control) to account for non-proteolytic mass loss (Figure 4).

Within the first day there was mass loss even in PBS (approx. 20%) due in part to drug elution from the films (drug loading accounts for <10%) and release of noncrystallized soluble silk into the release buffer (Figure 4). For films that are not loaded with EDTA (adenosine alone and control films), there was a significant increase in mass loss for films in protease type XIV compared to films in the PBS control. While control films lost only 20.7% of their initial mass after 5 days in PBS, control films in protease type XIV lost an average of 61.4% of the mass within 24 hours, and were fully degraded by day 4 (100% loss of initial mass). Adenosine films lost an average of 63.1% of their mass after 5 days in protease type XIV (compared to 15.8% mass loss in PBS). Both films loaded with EDTA (EDTA alone and EDTA+adenosine) exhibited much less difference in mass lost in PBS controls compared to the proteinase type XIV treatments (Figure 4). After 5 days in release buffer, EDTA films lost 14.2% of their initial mass in PBS compared to 21.4% loss of initial mass in protease type XIV. After 5 days in release buffer, EDTA+adenosine films lost 15.3% of their initial mass in PBS compared to 19.7% loss of initial mass in protease type XIV. Mass loss in PBS versus proteinase type XIV was not statistically significant for EDTA alone, but was statistically significant for all other groups (two tailed t-test, $df = 4$, $p < 0.05$). Silk films loaded with EDTA that released EDTA into the buffer once the films were immersed (EDTA concentration in the buffer after 3 days was 0.58 ± 0.04 mg/mL, or approx. 2 mM, above the 1–10 μ M effective concentration [49]), exhibited less mass loss over 5 days in protease type XIV than films that were not loaded with EDTA. These data suggest that the EDTA-releasing silk films were capable of inhibiting proteolytic degradation in protease type XIV.

3.3 EDTA and adenosine release from silk encapsulated reservoirs in PBS and protease type XIV release buffer

Since proteolysis increased drug release from the silk carriers and EDTA release from the silk films inhibited proteolysis, the effect of the combination of sustained EDTA release

from silk carriers on proteolytic activity and the release of the small molecule anticonvulsant adenosine from encapsulated reservoirs was investigated. The cumulative release of EDTA from encapsulated reservoirs containing 50% adenosine and 50% EDTA in PBS was assessed in 0.1 mg/mL protease type XIV (Figure 5).

EDTA release was sustained for 5 days. Linear zero-order release was observed for the first three days ($R^2_{\text{PBS}} = 0.97$, $R^2_{\text{Protease}} = 0.99$) followed by decreased release for an additional two days. The cumulative release of adenosine from encapsulated reservoirs containing 50% EDTA and 50% adenosine (ETDA + Adenosine) and encapsulated reservoirs containing 50% mannitol and 50% adenosine (Mannitol + Adenosine) was assessed in PBS (controls) and in 0.1 mg/mL protease type XIV solution (Figure 6). The release of adenosine was slower and consequently exhibited longer release duration than EDTA (Figure 5) despite the same initial reservoir coatings and initial drug loading. This is attributed to the lower water solubility of adenosine compared to EDTA. The curves in Figure 6 show that release of adenosine from reservoirs containing 50% mannitol/50% adenosine and reservoirs containing 50% EDTA/50% adenosine were similar in PBS, suggesting that EDTA can be incorporated into reservoirs without affecting adenosine release and that any differences observed between release profiles in PBS and in 0.1 mg/mL protease type XIV are due to differences in proteolytic activity related to film degradation. The proteinase increased drug release from both types of reservoirs, while EDTA release significantly reduced the effects of proteolysis on release rates. For a 10 day long release study the reservoirs comprised of EDTA+adenosine exhibited statistically significant different release rates for only 4 out of 10 days while the mannitol+adenosine reservoirs exhibited statistically significant different release rates for 8 out of 10 days (two tailed t-test, $df = 4$, $p < 0.05$). The increase in adenosine release rate was caused by proteolytic degradation and inhibited by sustained release of EDTA, reflected in the decrease in release duration (represented by t_{50}) and decrease in linearity of release (represented by decreased values of R^2 and n) for reservoirs containing mannitol and adenosine compared to reservoirs containing EDTA and adenosine (Table 1).

3.4 Effect of EDTA release from silk encapsulated reservoirs on protease type XIV activity

To characterize the effects of sustained release of EDTA from silk encapsulated reservoirs on proteolytic activity, the enzyme activity was assayed in collected samples (Figure 7). PBS samples exhibited no proteolytic activity. Every 24 hours proteinase was replaced with fresh 0.1 mg/mL protease solution. Fresh, unincubated 0.1 mg/mL proteinase solution assayed prior to study was taken as the value for Day 0/100% proteinase activity.

Even in the absence of inhibitor, some reduction of activity, up to 40%, was observed for encapsulated reservoirs containing 50% mannitol and 50% adenosine and reservoirs containing 100% mannitol. However, protease solutions must be changed daily due to activity loss at 37°C incubation [10]. The reservoirs containing 50% EDTA and 50% adenosine exhibited a significant reduction in proteinase activity for 5 days. After 5 days, the proteinase activity returns to levels comparable to non-EDTA containing formulations. The concentration of EDTA (in mg/mL) in the release buffer versus proteinase activity (in $\mu\text{g/mL}$ of active proteinase type XIV) is shown in Figure 8.

On day 1 when EDTA release was highest, protease type XIV was almost entirely inhibited. During the first 5 days while EDTA release was sustained, proteinase activity was reduced compared to proteinase activity levels observed when EDTA release subsided. In the absence of EDTA protease type XIV exhibited approx. 60–100% of initial activity. Overall, the concentration of EDTA released from the reservoirs proportionally inhibits the activity of protease type XIV present in the release buffer.

4. Discussion

Understanding and controlling the degradation behavior of silk is important for implantable medical applications. The degradation behavior has been studied *in vitro* and *in vivo* (Table 2A,B) and reviews of the process have also been published [9;57]. These studies have shown that the degradation behavior of silk is dependent on many factors including silk processing (organic solvent vs. aqueous processing related to crystalline content, silk concentration and porosity [11]), silk material format [50–51], type and concentration of enzyme [50] and immune system response [11].

The rate of drug release from implantable biodegradable systems is dependent on the diffusion of the drug through the polymer, degradation of the polymer carrier, or a combination of both mechanisms [27]. The rates of drug diffusion from a polymer carrier and degradation of the polymer carrier are affected by both the intrinsic properties of the drug, the polymer carrier and the external environment [58]. Release rate can be controlled via carrier morphology (geometry, number/thickness of coating [59,60]), but for many applications implant geometry may be constrained. Drug properties can also be manipulated for controlled release (PEGylation to increase apparent drug size [61], manipulation of aqueous solubility through salt formation [62]) but these approaches increase system complexity and must be screened to ensure they do not diminish or alter efficacy of the therapeutic to be delivered.

Manipulation of polymer properties (molecular weight of PLGA [59], degree of cross-linking of collagen [25], beta-sheet content of silk [16,21–22]) to control release is a common approach to regulate release rates because these are relatively simple yet effective options *in vitro* and result in systems with predictable and controlled release behavior. However, external environmental properties including pH, temperature and enzymes [58] can impact drug release kinetics and polymer degradation *in vivo*. This is particularly the case for natural biomaterials like silk, collagen and HA that are subject to enzymatic degradation. Despite the substantial potential impact of the external environment on these processes, manipulation of environmental properties to control drug delivery has not been extensively explored. The inclusion of controlled release of drugs to mediate local immune responses to implants has been proposed (63–65), which suggests localized drug delivery can be used to modulate environmental conditions at the site of implantation. The concept of using controlled release of inhibitors to regulate proteolytic degradation and drug delivery from silk carriers was inspired in part by the natural system. The silkworm, *Bombyx mori*, produces a protease inhibitor in the silk gland which is sequestered within the cocoon to protect against premature proteolytic degradation during development [66].

EDTA was chosen as a model inhibitor for the present study due to its broad specificity and general safety. EDTA inhibits a wide range of proteolytic enzymes including metalloproteinases and calcium dependent cysteine proteinases by chelating the metal ions required for enzymatic activity [49,67]. The major disadvantage of using metal chelators for proteinase inhibition is that they are non-specific and can interfere with other metal ion-dependent biological processes. However, the broad specificity of EDTA is suitable to demonstrate proof-of-principle and might be advantageous for implantation sites where the proteinase composition is unknown and cannot be specifically targeted.

EDTA use in humans is considered to be safe and has been used for decades as a common additive of the food-industry to preserve freshness [68]. Medically, EDTA is used for acute, high dose lead poisoning [69–71]. EDTA chelation has also shown promise in treating medical conditions requiring excess calcium clearance, including calcific tendinitis of the shoulder [72] and vascular disease [73–75]. Chelation of Cu^{2+} and Zn^{2+} ions has been

proposed as a potential treatment for Alzheimer's Disease as they are believed to contribute to amyloid beta deposition [76]. EDTA has also been shown to enhance the efficacy of various antimicrobials and anti-parasitics [77–79]. Several metalloproteinase inhibitors (including CaNa_2EDTA) have been shown to significantly reduce tissue damage caused by viper venom by inhibiting the activity of ECM-degrading enzymes present in the venom [80–81], which demonstrates that site-specific EDTA delivery can effectively inhibit local proteinase activity.

Protease type XIV, also called Pronase E and Actinase E, is a mixture of proteolytic enzymes produced by *S. griseus*. Protease type XIV was selected for study as it is often used for *in vitro* modeling of proteolytic degradation of silk biomaterials (Table 1A), has broad specificity [82] and degrades silk more aggressively than other candidate proteinases including collagenase and α -chymotrypsin [56,45,50]. Because this approach was effective for one of the most aggressive silk-degrading proteinase, it could also be applied to other proteinases and their inhibitors or activity enhancers.

Co-loading enzyme inhibitors into drug delivery systems allows for either non-specific suppression of local enzymatic degradation (resulting in release behavior *in vivo* that more closely resemble predictive *in vitro* models) or specific, targeted enzyme inhibition. Studies have shown that small molecule inhibitors, peptidomimetic inhibitors and proteinase-binding antibodies can effectively selectively suppress local enzymatic degradation [80–81]. Further, these different inhibitors exhibit different efficacies, suggesting that varied inhibition strategies can be used to achieve different enzymatic degradation profiles [80–81]. Previous work has also demonstrated that different proteinases achieve varied silk degradation effects (α -chymotrypsin, for example, degrades random coils and alpha-helices but not the beta-sheets [83]) Specific proteinase inhibition profiles could potentially be used to precisely control the *in vivo* degradation of silk implants by manipulating which local proteinases are active or inactive and to what extent. This strategy would be applicable to other drug carriers such as collagen and HA which, like silk, are susceptible to enzymatic degradation. This potential to tightly control local degradation represents a controllable feature for enzymatically degraded biomaterials that cannot easily be designed into drug release systems based on hydrolytically degradable polymer systems, such as polyesters like PLGA and PCL.

5. Conclusions

Increased proteinase concentrations increased the rate of drug release from silk carriers. Loading EDTA into silk carriers inhibited proteolytic degradation of silk films and delayed drug release from silk encapsulated reservoirs immersed in proteinase solution. These data demonstrated the effects of proteolytic degradation on drug release from silk carriers and introduced a new strategy to control degradation rate via manipulation of local proteolytic activity. Future work includes identifying strategies to achieve sustained release of silk-specific proteases or proteinase-activity enhancers from the carriers themselves to increase local degradation rates. The modulation of local enzyme function via-controlled release could be applied to a range of silk material formats, including drug delivery implants and tissue engineering scaffolds.

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Figure 1. Chemical structures of (A) reactive red 120 (MW = 1469.98) (B) indigo carmine (MW = 466.35) (C) adenosine (MW = 267.24) and (D) ethylenediaminetetraacetic acid (EDTA) (MW = 292.24)

Figure 2.

(A) Cumulative release of reactive red-120 from 8% (w/v) silk films in PBS and varying concentrations of proteinase type XIV. (B) Average daily release rate of reactive red 120 from films (in % total load per day) versus protease type XIV concentration in the release buffer. N=4, error bars represent standard deviations (where error bars aren't shown they fall into background).

Figure 3.

(A) Cumulative release of reactive indigo carmine from 8% (w/v) silk films in PBS and varying concentrations of protease type XIV. N=4, error bars represent standard deviations (where error bars aren't shown they fall into background). (B) Siepmann-Peppas release coefficient (n) versus protease type XIV concentration in the release buffer

Figure 4.

Mass loss over 5 days of silk films immersed in PBS (solid lines) or 0.1 mg/mL proteinase type XIV solution (dashed lines). N=3, error bars represent standard deviation. Where error bars are not shown, the standard deviation falls into the background.

Figure 5.

Cumulative EDTA release (in percent total load, approx. 35 mg) from silk encapsulated reservoirs in PBS (solid line) and 0.1 mg/mL proteinase type XIV (dashed line) at 37°C. N=3, error bars represent standard deviations. Where error bars are not shown they fall into background.

Figure 6.

Cumulative adenosine release from silk encapsulated reservoirs in PBS (solid lines) and 0.1 mg/mL proteinase type XIV (dashed lines) at 37°C. Filled diamonds indicate release of adenosine from 50% EDTA and 50% adenosine encapsulated reservoirs, empty circles indicate release of adenosine from 50% mannitol and 50% adenosine encapsulated reservoirs. N=3, error bars represent standard deviations. Where error bars are not shown they fall into background.

Figure 7.

Activity of proteinase type XIV release buffers for encapsulated reservoirs containing 50% EDTA /50% adenosine (diamond with dashed line), 50% mannitol/50% (squares with dotted line) or 100% mannitol (circle with solid line). N = 3, error bars represent standard deviations. Where error bars are not shown they fall into background.

Figure 8.

Concentration of EDTA released from 50% EDTA/50% adenosine encapsulated reservoirs (in mg/mL) versus active proteinase XIV in release buffer (in µg/mL). N=3, error bars represent standard deviations. Where error bars are not shown the standard deviation falls into background.

Table 1

Data for cumulative adenosine release from encapsulated reservoirs containing either 50% adenosine and 50 % mannitol or 50% EDTA and 50% adenosine in either PBS or 0.1 mg/mL protease type XIV

	Mannitol + Adenosine (no inhibition of proteolysis)		EDTA + Adenosine (inhibition of proteolysis)	
	PBS	0.1 mg/mL Protease type XIV	PBS	0.1 mg/mL Protease type XIV
Time at which 50% total adenosine load is released (t_{50})	3.04 days	1.8 days	3.35 days	2.77 days
Correlation Coefficient (R^2)	0.93	0.71	0.94	0.91
Siepmann-Peppas Release Coefficient (n)	0.721	0.493	0.776	0.676

Table 2

A. Degradation behavior of silk <i>in vitro</i>			
Proteinase	Material Format	Degree and Measure of Degradation	Ref.
Collagenase type F	Silk film	3.8% and 10.7% mass loss at days 1 and 17, respectively	[50]
	0.5 mm thick freeze-dried hydrogel sheet	Mass loss of ~52% after 15 days (compared to ~32% mass loss in PBS); internal pore size increased with increasing degradation time (SEM)	[45]
α -chymotrypsin type I-S	Silk film	9% and 16.5% mass loss at days 1 and 17, respectively	[50]
Protease Type XIV from <i>Streptomyces griseus</i>	0.5 mm thick freeze-dried hydrogel sheet	Mass loss of ~70% after 15 days (compared to ~32% mass loss in PBS); internal pore size increased with increasing degradation time (SEM)	[45]
	Silk encapsulated solid reservoirs (silk film)	Extensive degradation of ~0.3 mm thick silk coating by day 5, total degradation by day 7; increased surface roughness for both ~0.3 mm and ~0.8 mm coatings (SEM)	[22]
	Silk yarn	Fibroin filament diameter decreased exponentially with time in protease to 66% of initial diameter at 10 weeks; greater than 50% mass loss after 42 days	[14]
	Silk nerve guide conduits	~60% mass loss after 9 days, ~70% mass loss after 18 days	[51]
	Degummed silk fibers	No mass loss after 7 days, ~19% mass loss after 18 days	[51]
	Aqueous/NaCl derived porous sponges	~54.5% and ~88.6% mass loss by day 1 and day 4, respectively	[52]
		~95% mass loss by day 7	[53]
	HFIP/sucrose-derived porous sponges	~17.8% and ~35.2% mass loss by day 1 and day 4, respectively	[52]
	Autoclaved HFIP/sucrose-derived porous sponges	~7% mass loss by day 7	[53]
Protease type XXI from <i>S. griseus</i>	Silk film	35% and 64.3% mass loss at days 1 and 17 respectively	[50]
	Silk fiber	Negligible mass loss, but drop in strength and elongation observed relative to increased degradation time	[50]
Protease type XXIII from <i>S. griseus</i>	Silk film	Evidence of proteolytic degradation (decreasing thermal stability and change in secondary structure with incubation time)	[54]
Protease type XXV from <i>S. griseus</i>	Silk film	~10% weight loss over 5 days (compared to approx. 2% loss in PBS); increased surface roughness (SEM)	[55]
Crude papain	Freeze-dried regenerated fibroin solution	Degradation after 60 minute incubation confirmed by tricine SDS-PAGE	[56]

B. Degradation behavior of silk <i>in vivo</i>			
Implantation Site	Material Format	Degree and Measure of Degradation	Ref.
Rat; brain (hippocampus)	Silk-coated porous sponges imbedded with microspheres	Histology sections of silk sponges exhibited on average 9.8% more surface area after 4 weeks <i>in vivo</i>	[24]
Rabbit; subcutaneous near the spinal column	Silk nerve guide conduits	25, 65.4 and 79% mass loss at 8-, 12- and 24- weeks post implantation, respectively	[51]
	Degummed fibers	10 and 34% mass loss at 12- and 24- weeks post-implantation, respectively	[51]

B. Degradation behavior of silk <i>in vivo</i>			
Implantation Site	Material Format	Degree and Measure of Degradation	Ref.
Lewis rats, subcutaneous	Aqueous/NaCl derived porous sponges	Degraded to completion between 2 and 6 months	[11]
	HFIP-derived porous sponges	Degradation visible in histology sections after 6 and 12 months of implantation	[11]
Lewis rats and nude rats, intramuscular	Aqueous/NaCl derived porous sponges	After 8 weeks, loss of structural integrity, most pores collapsed; cross-sectional area of scaffolds in Lewis rats ~1/3 of that in nude rats (indicating immune-system mediated degradation)	[11]