Physical and Chemical Aspects of Stabilization of Compounds in Silk

Eleanor M. Pritchard[1], **Patrick B. Dennis**[2], **Fiorenzo Omenetto**[1], **Rajesh R. Naik**[2], and **David L. Kaplan**[1,*]

[1]Department for Biomedical Engineering, Tufts University, Medford, MA 02155, USA

[2]Nanostructured and Biological Materials, Materials and Manufacturing Directorate, Wright-Patterson AFB OH 45433, USA

Abstract

The challenge of stabilization of small molecules and proteins has received considerable interest. The biological activity of small molecules can be lost as a consequence of chemical modifications, while protein activity may be lost due to chemical or structural degradation, such as a change in macromolecular conformation or aggregation. In these cases stabilization is required to preserve therapeutic and bioactivity efficacy and safety. In addition to use in therapeutic applications, strategies to stabilize small molecules and proteins also have applications in industrial processes, diagnostics, and consumer products like food and cosmetics. Traditionally, therapeutic drug formulation efforts have focused on maintaining stability during product preparation and storage. However, with growing interest in the fields of encapsulation, tissue engineering and controlled release drug delivery systems, new stabilization challenges are being addressed; the compounds or protein of interest must be stabilized during: (1) fabrication of the protein or small molecule loaded carrier, (2) device storage, and (3) for the duration of intended release needs *in vitro* or *in vivo*. We review common mechanisms of compound degradation for small molecules and proteins during biomaterial preparation (including tissue engineering scaffolds and drug delivery systems), storage and *in vivo* implantation. We also review the physical and chemical aspects of polymer-based stabilization approaches, with a particular focus on the stabilizing properties of silk fibroin biomaterials.

1. Introduction

Traditionally, stabilization approaches include modification of the small molecule or protein to reduce susceptibility to degradation (including chemical and genetic engineering, cross-linking, protein crystallization, and chemical modification), optimization of the storage environment (lyophilization, storage in organic solvents, freezing, storage in appropriate containers to prevent light or oxygen exposure) or additives (buffers to optimize pH, antioxidants to limit oxidation). Controlled release systems offer several advantages over conventional drug administration approaches, including enhanced efficacy and cost-efficiency, reduction or elimination of unwanted side-effects, reduced frequency of

*Corresponding author: David L. Kaplan Tufts University Department of Biomedical Engineering 4 Colby St. Medford, Massachusetts 02155 U.S.A. Tel: 617-627-3251 Fax: 617-627-3231, david.kaplan@tufts.edu.

administration, improved patient convenience and compliance and drug levels that are continuously maintained in a therapeutically desirable range (Langer, 1980). Despite their therapeutic promise, controlled release systems introduce new stabilization challenges. Many proposed carrier fabrication methods feature harsh conditions that can damage or denature the incorporated protein or compound, including the use of organic solvents, elevated temperatures, toxic cross-linking chemicals and vigorous agitation (Mehta *et al.*, 2011; Fu *et al.*, 2000). One of the most commonly investigated biodegradable polymers, poly-(DL-lactide-co-glycolide) (PLGA), degrades via hydrolysis to acidic products, producing low pH microclimates detrimental to drug stability (Zhu *et al.*, 2000; Varde and Pack, 2007). During in vitro and in vivo release, reservoir systems encounter conditions detrimental to stability: high-concentration, hydration, elevated temperature (37°C) and extended timeframes (potentially months) (Fu *et al.*, 2000; Bilati *et al.*, 2005). For controlled and sustained release biomaterials, stabilization must therefore be achieved and maintained during: (1) fabrication of the protein or small molecule loaded carrier, (2) device storage, and (3) for the duration of intended release needs *in vitro* or *in vivo*.

While some of the techniques employed for conventional formulation can potentially be applied to polymer drug carriers (for example the inclusion of antacids in PLGA carriers to neutralize acidic degradation products (Zhu *et al.*, 2000; Varde and Pack, 2007)), biomaterials systems which can themselves effectively stabilize proteins and small molecules would have several important biomedical applications. Improved stabilization during storage and sustained-release from drug carriers would improve the therapeutic efficacy and safety of unstable drugs like antibiotics and chemotherapeutics, particularly in developing-world clinical settings where refrigeration is limited. Immobilization of bioactive dopants like indicator dyes, enzymes and immunological proteins could potentially be built into analytical and diagnostic applications (Lawrence *et al.*, 2008; Tsioris *et al.*, 2010; Omenetto and Kaplan, 2008; Omenetto and Kaplan, 2010). Stabilization of enzymes used in industrial processes via polymer carriers would allow cost-efficient re-use of expensive enzymes or allow expanded utility in a variety of sensor and diagnostic applications (Saxena et al., 2010; Mateo et al., 2007). Stabilization of signaling molecules like growth factors and cytokines within tissue engineering scaffolds would enhance therapeutic outcomes (Chen et al., 2010b; Biondi et al., 2008). Given the nature of the potential applications, the need to develop platforms based on biocompatible, biodegradable materials is critical. A variety of natural polymers have been proposed for immobilization including cellulose, cross-linked dextrans, starch, tannin, agarose, chitin, chitosan, collagen, gelatin and albumin (Zhang, 1998; Acharya *et al.*, 2008). However, no methods to date provide for immobilization in polymer biomaterial systems with the necessary combination of simplicity, versatility, tunability, biocompatibility and stabilizing effects on encapsulants.

Silk fibroin is a biologically-derived protein polymer purified from domesticated silkworm (*Bombyx mori*) cocoons that possesses many attractive features for immobilization. Silk exhibits excellent mechanical properties, biocompatibility (Leal-Egaña and Scheibel, 2010; Meinel *et al.*, 2005; Panilaitis *et al.*, 2003), optical properties (Lawrence et al., 2008; Omenetto and Kaplan, 2008) and biodegrades to non-toxic products via proteolysis (Wang *et al.*, 2008a; Horan *et al.*, 2005). Silk is edible, non-toxic and relatively inexpensive: Qian

et al. successfully immobilized glucose oxidase using regenerated silk fibroin from the waste silk of a silk mill (Qian *et al.*, 1996). A broad range of useful silk material formats can be prepared using mild, simple processes (Numata and Kaplan, 2010; Pritchard and Kaplan, 2011). The ambient, aqueous processing options utilized during silk protein preparation and materials fabrication represents a major advantage over other immobilization materials in that stable silk matrices can be prepared without conditions which can potentially degrade incorporated proteins and small molecules such as harsh temperature, pressure or chemicals (Liu *et al.*, 1996; Qian *et al.*, 1997; Demura *et al.*, 1989; Lu *et al.*, 2010). Unlike many biological derived proteins, silk is inherently stable to changes in temperature and moisture (Kuzuhara et al., 1987; Omenetto and Kaplan, 2010) and mechanically robust (Altman et al., 2003). The unique block copolymer structure of silk, consisting of large hydrophobic domains and small hydrophilic spacers, promotes self-assembly into organized nanoscale crystalline domains (β-sheets) separated by more flexible hydrophilic spacers. Proteins loaded in silk matrices may form hydrogen bonds with the silk fibroin protein, promoting protein stability. As a result, assembled silk fibroin offers a highly stabilizing environment for incorporated proteins and small molecules (Lu *et al.*, 2009) providing the appropriate "molecularly crowded" environment for enzyme stabilization.

2. Silk based strategies to prevent chemical and structural degradation

Silk-based immobilization strategies to improve stability of compounds or proteins generally fall into four main categories. Note that these methods can also be combined. The choice of which approach or combination of approaches to use depends on the application and the properties of compound or protein being stabilized.

(1) Adsorption

Adsorption refers to attachment to the surface of the silk carrier by weak forces, such as van der Walls or hydrophobic interactions. Simple adsorption can be used to decorate silk surfaces with proteins or compounds of interest (Vepari and Kaplan, 2007). Adsorption has the advantages of mild processing, relatively ease and low cost. However, the extent of compound or protein immobilization will depend on the strength of the interaction between the compound or protein of interest and the silk.

(2) Covalent attachment

Silk fibroin can be functionalized using the amino acid side chain chemistry, particularly carbodiimide and diazonium chemistries, which uses amine or carboxyl groups on silk for modification. These modifications provide chemical handles for the covalent attachment of proteins and compounds to silk carriers, producing a stronger interaction between compound or protein and silk matrix compared with adsorption. Chemical reactions that have been used to modify the amino acids in silk proteins for various biomedical applications including conjugation of compounds and proteins to silk fibroin biomaterials have been reviewed elsewhere (Murphy and Kaplan, 2009).

(3) Entrapment in carriers

> Entrapment (or "bulk-loading" as it is termed to in the field of drug delivery) refers to capture of the protein or compound of interest by mixing into the silk solution prior to material format preparation. Bulk loading most commonly involves the casting of films of the desired surface area and thickness from solutions of compound or protein and aqueous silk fibroin followed by drying and post-treatment to produce the desired material properties (Hofmann et al., 2006), while other silk material formats and processing options have also employed this method. Unlike chemical coupling, the protein or compound of interest is not covalently attached to the silk protein, but entrapment and encapsulation offer the advantage of isolating most of the loaded compound or protein from the bulk medium. Entrapment in silk film matrices is inexpensive, straightforward and applicable to a wide range of compounds or proteins. In addition, proteins or compounds of interest can be entrapped in silk carriers using mild, ambient, aqueous manufacturing conditions, preserving their bioactivity (Liu *et al.*, 1996; Qian *et al.*, 1997; Demura *et al.*, 1989a; Hofmann *et al.*, 2006; Lu *et al.*, 2009; Lu *et al.*, 2010). Bulk-loaded silk films can also be prepared such that the water-solubility of the silk film is retained (Kim et al., 2009; Kim et al., 2010). This allows rapid recovery of compound loaded into silk storage matrices in applications where sustained release or long-term immobilization is not optimal. On the other hand, water insoluble silk carriers can be used for sustained release applications and applications where silk supports are required for longer duration (e.g. bone regeneration).

(4) Encapsulation in micro- or nano-particles

> Comparable to entrapment, encapsulation refers to loading of a compound or protein of interest into silk micro- or nano-scale particles. For reviews of silk micro and nano-particle encapsulation, see Pritchard and Kaplan, 2011; Wenk et al., 2011 and Numata and Kaplan, 2010.

3. Protein and Peptide Stabilization by Silk

3.1. Enzymes

> Stabilization of enzymes using silk biomaterials (particularly bulk-loaded films) has been reported for various enzymes (including horseradish peroxidase (HRP), glucose oxidase (GOx) and lipase) for industrial, medical, diagnostic and biosensor applications (Table 1). Note that the functionality of biosensors based on enzymes immobilized in silk film can be further improved by increasing matrix permeability to substrates, for example, through control of the secondary structure/beta-sheet content (Demura *et al.*, 1989) or the addition of porogens like PEG, which improve diffusion of substrates through the membrane (Demura *et al.*, 1991; Liu *et al.*, 1996).

> **3.1.1. Horseradish peroxidase (HRP)**—Horseradish peroxidase (HRP) is widely used as an indicator enzyme in enzyme immunoassays, enzyme electrodes, effluent treatment and synthetic organics, but lacks stability in solution (Lu *et al.*, 2009). The addition of silk solution to an HRP solution increased enzyme activity 30-40% and increased the half-life of HRP stored at room temperature to 25 days (compared with 2.5 hours for HRP in buffer alone) (Lu *et al.*, 2009). Several authors have demonstrated that bioactivity of HRP is

preserved when entrapped in silk films (Demura et al., 1989b; Qian *et al*., 1996; Qian *et al*., 1997; Hofmann *et al*., 2006; Lu *et al*., 2009; Lu *et al*., 2010). HRP activity was also preserved when incorporated via bulk-loading into silk optical gratings (Lawrence *et al*., 2008; Omenetto and Kaplan, 2008) and silk microneedle arrays (Tsioris *et al*., 2011), and when encapsulated in silk microspheres by means of a phospholipid template process (Wang *et al*., 2007). After 5 months of storage, HRP immobilized in silk films retained 24%, 22%, 17% of the initial activity when stored at 4°C, room temperature and 37°C, respectively. Stabilization was higher in untreated films than methanol treated films and higher in films with higher loading compared with low loading (Lu *et al*., 2009). The response of hydrogen peroxide sensors based on HRP immobilized in silk films only decreased 16% after 2 months of storage at 4°C (Qian *et al*., 1996). Lu *et al*. report retention of more than 90% of the initial enzymatic activity for HRP immobilized in silk films after 2 months at 37°C (Lu *et al*., 2010)

Vepari and Kaplan generated stable immobilized HRP gradients within three-dimensional silk fibroin sponges using either carbodiimide coupling of activated HRP or adsorption of HRP onto the silk substrate. After a 30 min incubation at 60°C, free enzyme lost all activity; compared to approx. 75% and 25% activity remaining (normalized to HRP activity at 25°C) for covalently coupled and adsorbed HRP, respectively. After 30 min incubation at 37°C, activity of HRP covalently coupled to silk sponges increased above 100%, consistent with the findings of Lu *et al*. that immobilization in silk can enhance apparent enzyme activity (possibly through renaturation of denatured protein) (Lu *et al*., 2009). A scaffold loaded with HRP via adsorption was incubated for 4 days in PBS solution to verify gradient stability; some desorption was observed but the gradient pattern was found to be intact. The simple, mild processes described for gradient immobilization of HRP onto silk sponges could be extended to a variety of proteins or small molecules (Vepari and Kaplan, 2006).

3.1.2. Glucose oxidase (GOx)—Glucose oxidase (GOx) is another important enzyme for biosensor applicationa. Several authors have demonstrated that immobilization of GOx in silk films dramatically improves the enzymes thermal and pH stability (Demura *et al*., 1989a; Kuzuhara *et al*., 1987; Zhang *et al*., 1998a; Lu et al., 2009). Demura *et al*. reported that GOx immobilized in fibroin films retained more than 90% relative activity after soaking in phosphate buffer at pH 7 at 4°C for 4 months. At 50°C, free enzyme loses 30% activity, while the relative activity of the immobilized enzyme scarcely changed (Demura *et al*., 1989). Kuzuhara *et al*. reported that immobilized GOx on fibroin retained 100% and 97% relative activity after 20 min incubation at 60°C and 70°C, respectively, compared with total activity loss above 6°C for free enzyme (Kuzuhara *et al*., 1987). GOx-immobilized in methanol treated silk films exhibited high storage stability: glucose detecting ability was retained for over 2 years in films stored at 4°C. Enzymatic activity was also maintained when GOx loaded silk films were immersed in buffers with varied pH (range of 5.0-10.0), exposed to temperatures from 20-50°C and used repeatedly for over 1,000 detections (Zhang *et al*., 1998a). GOx immobilized in silk films and blended silk-poly(vinyl alcohol) films exhibited improved pH stability and thermal stability compared with free GOx and GOx immobilized via glutaraldehyde crosslinking onto carbon electrodes. After 30 min incubation at 50°C, enzyme immobilized in silk membranes retained 93% activity, enzyme

immobilized in silk-PVA blend films retained 86% activity and free enzyme activity dropped to 30% (Liu *et al*., 1996). Immobilization of GOx in silk fibroin not only improved thermal stability compared with free enzyme, but also improved stability compared with enzyme immobilized in gelatin films, which were unstable at elevated temperatures (Kuzuhara *et al*., 1987).

Perhaps most notably, Lu et al. report that for untreated films loaded with 1 wt% GOx, films stored at 4°C and 25°C exhibit slight activity loses during the first 3 months of storage, then during the remaining five months of storage, GOx activity increases above 100% of the initial activity. The authors attribute this observation to reversible denaturation of GOx in the original solution followed by renaturation upon interaction with the silk material. The authors also report that GOx stability is higher in untreated films compared with methanol treated films, possibly due to the optimal hydrophobic interactions between the GOx and silk allowing the enzyme to undergo structural changes (i.e. renaturation), as opposed to the more restrictive, highly hydrophobic and stacked β-sheet structures characteristic of methanol treated films (Lu et al., 2009).

3.1.3. Other enzymes—Lipase immobilized on silk fibers has successfully been used for hydrolysis of sunflower oil (Chatterjee *et al*., 2009) and hydrolysis of sunflower oil for the production of fatty acids (Chen *et al*., 2010a). Lipase immobilized by adsorption on woven silk fibers showed enhanced pH and temperature stability compared with soluble lipase (Chen *et al*., 2010a). Lipase immobilized on silk fibers via cross-linking with glutaraldehyde was stable for 2 months at 4°C (Chatterjee *et al*., 2009). At room temperature, activity of both the immobilized and free lipase declined, but the relative stability of the immobilized lipase was significantly higher than the free lipase. The crosslinking of lipase onto silk fibers resulted in the loss of 12% of activity, but the immobilized lipase could be reused even up to the third reaction cycle (Chatterjee *et al*., 2009). Cholesterol oxidase (ChOx) covalently immobilized onto fibrous, porous woven silk mats achieved remarkably high storage and operational stability. The half life (time at which 50% of initial activity remained) of the immobilized ChOx stored in a closed container at 4°C without being used was nearly 13 months (Saxena *et al*., 2010). Lu *et al*. reported significant activity retention for enzymes (lipase, glucose oxidase and horseradish peroxidase) entrapped in silk films over 10 months even at 37°C, and observed a correlation between silk protein processing and enzyme stability, suggesting that stability could be optimized via processing (Lu *et al*., 2009).

Silk fibroin has also proven to be an excellent substrate for tyrosinase immobilization, leading to higher conversion rates, high storage, pH and thermal stability and greater reusability. Under the same storage conditions, free enzyme lost all activity within 5 days, while immobilized enzyme retained approximately 75% of its original activity even after 10 days (Acharya *et al*., 2008). Ribonuclease immobilized onto woven silk substrates using diazo-coupling retained 63% of its activity after 7.2 months of storage in 0.1 M NaCl at 0-4°C (Cordier *et al*., 1982). Preparations of alkaline phosphatase immobilized on woven silk by covalent (diazo, azide, or glutaraldehyde) or adsorption methods retained bioactivity (Grasset *et al*., 1983). Stability of β-glucosidase entrapped in ethyl alcohol-treated silk films was protected against heating, electrodialysis and protease treatment (Miyairi *et al*., 1978). After 30 min incubation at 55°C, soluble enzyme lost approx. 50% of the initial activity,

while immobilized enzyme lost only 10%. Exposure to papain reduced activity of soluble enzyme, but did not affect immobilized enzyme (Miyairi *et al.*, 1978). An amperometric urate sensor based on uricase immobilized in silk fibroin membrane could be used repeatedly (more than 1,000 times), stored for over 2 years, and was stable in phosphate buffer for 3-4 months (Zhang *et al.*, 1998b). Four heme-proteins (myoglobin, hemoglobin, horseradish peroxidase, and catalase) incorporated into regenerated SF films on graphite electrodes (GE) retained their ability to catalyze the reduction of hydrogen peroxide and nitric oxide. The investigation of UV–Vis and reflectance absorption infrared (RAIR) spectroscopy showed that the heme-proteins entrapped in silk maintained their native state and SEM and RAIR indicated that an intermolecular interaction existed between the heme-proteins and the fibroin (Wu *et al.*, 2006).

Organophosphorus hydrolase (OPH) is a bacterial enzyme which holds promise as an effective agent for the bioremediation of organophosphates. Due to their potent neurotoxicity in both insects and higher organisms, organophosphates are produced on an industrial scale as pesticides and chemical warfare agents (reviewed in Singh *et al.*, 2006). Regarding the later threat, coatings able to decontaminate and protect surfaces from nerve agent exposure while maintaining viability under a number of environmental conditions would be highly desirable. Towards this end, silk fibroin-entrapment of OPH has been tested for its ability to preserve enzymatic activity under hostile environmental conditions likely encountered by a surface coating. Silk fibroin-entrapped OPH is significantly stabilized during exposure to increased temperature and UV light when compared to the free enzyme (Dennis *et al.* in preparation). Additionally, the silk fibroin-entrapped OPH demonstrates remarkable tolerance to detergents and organic solvents. Thus, entrapment of OPH in silk fibroin not only increases enzyme stability under adverse environmental conditions, but also provides enzyme stability during the fabrications steps required to create surface coatings for decontamination and protection.

Silk-based enzyme stabilization approaches are also useful for stabilizing therapeutic enzymes, which might otherwise be difficult to safely and effectively administer. When the anti-leukemic enzyme L-asparaginase (ASNase) was coupled covalently with silk fibroin powder by glutaraldehyde, the conjugated enzyme exhibited enhanced thermostability, pH stability, storage stability and resistance to trypsin digestion. After 30 days at room temperature, unmodified enzyme only retained 20% of the original activity, while the fibroin conjugated enzyme retained more than 80% of the original activity. Further, covalent conjugation to silk powder increased the enzyme substrate affinity and circulating half-life compared with unmodified enzyme (Zhang et al., 2005). Phenylalanine ammonia-lyase (PAL) was entrapped in silk fibroin microparticles (less than 150 microns diameter). Entrapped PAL was more stable than lyophilized free PAL: the remaining activity after 82-days storage at 4°C was 75.4 and 34.4% for entrapped and free PAL, respectively. In vitro results showed that PAL entrapped in silk fibroin gained resistance to chymotrypsin and trypsin proteolysis. Following oral administration of free and entrapped PAL, plasma levels of PAL's reaction product, cinnamate, were monitored to assess the *in vivo* enzyme activity. The entrapped enzyme, but not the free form, caused a significant rise of plasma cinnamate,

suggesting the free enzyme lost bioactivity in vivo, but enzyme encapsulated in silk retained activity in the intestinal tract (Inoue et al., 1986).

3.2. Growth Factors

The goal of tissue engineering and regenerative medicine is the induction of therapeutic tissue repair or restoration of tissue function by using scaffold substitutes that recapitulate the microenvironments of the natural extracellular matrix (ECM) (Chen *et al*., 2010b). Providing bioactive signals such as cytokines and growth factors can enhance therapeutic outcomes. However, growth factor (GF) delivery via systemic injection is limited by short half-life, rapid diffusion from the injection site and (if delivery is not localized) systemic side-effects (Chen *et al*., 2007). GFs are also susceptible to degradation in vivo due to harsh proteolytic environments. In addition to providing sustained and localized delivery, immobilization of GFs in polymer carriers might improve stability (Chen *et al*., 2010b).

The native ECM is known to act as a protective storage depot for growth factors and releases them "on demand" (Benoit and Anseth, 2005; Wenk *et al*., 2010). Sakesela *et al* reported that soluble bFGF was readily degraded by plasmin, whereas bFGF bound to the ECM component heparan sulfate was protected from proteolytic degradation. Heparin sulfate (a highly anionic, sulfated glycosaminoglycan that binds numerous growth factors, including bFGF) acts as a natural, protective growth factor "carrier" (Sakesela *et al*., 1988). Release of ECM-bound growth factor is regulated by ECM-degrading proteinases expressed by normal and malignant cells (i.e. platelets, neutrophils, lymphoma cells) (Vlodavsky *et al*., 1991; Whitelock *et al*., 1996). Similarly, insulin-like growth factor I (IGF-I) is known to be entrapped in mineralized bone matrix during bone formation and actively released during bone remolding (Uebersax *et al*., 2008).

Based on this understanding of the native system, there is growing interest in biomimetic scaffolds that, like the ECM, support cell growth and also sequester and stabilize important bioactive signals like growth factors and cytokines (Chen *et al*., 2010b). In addition to providing superior physical support scaffolds for cell growth and proliferation (Wang *et al*., 2006), there is growing evidence that silk fibroin biomaterials possesses a remarkable ability to preserve the bioactivity of incorporated growth factors (Wenk *et al*., 2010), suggesting silk scaffolds could mimic the physiological role of the ECM, acting as storage for growth factors such as FGF-2, BMP-2 and VEGF, and releasing them in response to proteolysis. The mild, aqueous processing options for silk biomaterials described previously are well-suited to stabilization and delivery of fragile growth-factors.

In some cases, growth factor signaling may be enhanced when the growth factor is firmly attached to the biomaterial scaffold, whereas other growth factors achieve a stronger response in soluble form (Wenk *et al*., 2010). Accordingly, a range of silk immobilization strategies have been described. While storage stability for growth factor loaded silk biomaterials has not been reported, preservation of bioactivity (indicative of stabilization during fabrication and in vitro or in vivo release) has been extensively investigated (Table 2). Strong retention of growth factors by silk may be achieved by covalent coupling or, depending on the strength of the interaction between the growth factor and silk fibroin, simple adsorption or bulk loading.

When BMP-2 was applied to porous silk sponges, 25% of the initial BMP-2 was firmly adsorbed to the scaffold. The authors attributed enhanced mineralization at the center of silk biomaterial sponges to retention of BMP-2 at the center of the sponge, suggesting absorption onto silk enhanced stability compared to soluble BMP-2 (Karageorgiou *et al*., 2006). Release behavior indicated that bFGF (Wongpanit *et al*., 2010) and NGF (Uebersax *et al*., 2007) also interacted strongly with silk fibroin. Wenk *et al*. enhanced retention of FGF-2 by silk by decorating fibroin with sulfonic acid moieties (analogous to the natural FGF-2-binding sulfated glycosaminoglycan heparan sulfate in the ECM). This approach led to more than 99% FGF-2 retained up to 6 days and the potency of the growth factor (evaluated via reduced metabolic activities of human bone marrow derived mesenchymal stem cells (hMSCs) and increased levels of pERK1/2) was exclusively retained when bound to films of sulfonated SF derivatives, demonstrating FGF-2 stabilization (Wenk *et al*., 2010).

Karageorgiou *et al*. compared immobilization of BMP-2 onto silk films via adsorption and covalent coupling. Adsorbed BMP-2 on silk films caused an increase in hMSC osteogenesis compared with controls (unmodified silk films), though less of an increase in hMSC osteogenesis than covalently coupled BMP-2 on silk films. Increased osteogenesis was also reported when hMSCs were seeded onto the surfaces of BMP-2-decorated films compared with hMSCs exposed to similar amounts of soluble BMP-2, which the authors attributed to enhanced growth factor stability and higher protein concentrations in the local microenvironment (Karageorgiou *et al*., 2004).

Bulk-loading has been used to prepare growth factor loaded porous silk sponges and silk microspheres, useful material formats for tissue engineering applications. The bioactivity of BMP-2 immobilized in electrospun silk nanofiber mats was preserved as evidenced by increased osteogenesis of hMSCs cultured for 31 days on BMP-2-loaded scaffolds compared with empty control scaffolds (Li *et al*., 2006). IGF-I loaded into highly porous three-dimensional silk scaffolds enhanced chondrogenic differentiation of hMSCs compared with hMSCs cultured on unloaded control silk scaffolds (Uebersax *et al*., 2008). Nerve growth factor (NGF)-loaded porous tube-shaped scaffolds for nerve guides prepared by freeze drying molds filled with aqueous solutions of NGF and silk supported PC12 cell differentiation with neurite outgrowth (Uebersax *et al*., 2007). In addition, the silk fibroin immobilization matrix was able to preserve IGF-I potency (Uebersax *et al*., 2008) and NGF potency (Uebersax *et al*., 2007) during methanol treatment of the scaffolds.

Bessa *et al*. encapsulated BMP-2, BMP-9 and BMP-14 in silk microspheres and confirmed the bioactivity of the eluted growth factor in vitro and in vivo (Bessa *et al*., 2010). Wenk *et al*. prepared loaded IGF-I into silk fibroin spheres using laminar jet break-up of aqueous solutions, followed by water vapor annealing. This mild encapsulation process resulted in preservation of bioactivity, as indicated by enhanced in vitro proliferation of MG-63 cells compared with controls (Wenk *et al*., 2008). Wang *et al*. incorporated silk fibroin microspheres loaded with BMP-2 and IGF-I into aqueous-derived silk porous scaffolds using a gradient process. Both growth factors formed deep and linear concentration gradients in the scaffolds and were shown to induce and control hMSC differentiation (Wang *et al*., 2009).

3.3. Other proteins and peptides

The silk-film based immobilization approaches described in the previous section can also be applied to the stabilization of other proteins, particularly immunological proteins for diagnostics and biosensors (Table 3). Low molecular weight Protein A (LPA) is a powerful immunological tool, but is highly thermally unstable in solution. The thermal stability of LPA immobilized in silk fibroin films was found to be higher than that of free LPA at high temperature based on immunoglobulin G (IgG)-binding affinity: at 120°C, fibroin immobilized LPS retained 50% of its relative IgG binding, while free LPA retains less than 20% (Kikuchi *et al.*, 1999). Silk film optical gratings doped with hemoglobin and stored for 4 days at room temperature were studied for function. Preservation of the oxygen binding functionality of hemoglobin entrapped and stored in silk films was confirmed with an optical transmission experiment (Lawrence *et al.*, 2008; Omenetto and Kaplan, 2008). Primary antibody immobilized and enriched at the surface of fibroin substrates was stored at 25°C in dry conditions for 4 weeks and found to retain approx. 40% of its secondary antibody binding activity (Lu *et al.*, 2011). Green fluorescent protein entrapped in silk fibroin films was show to retain its nonlinear optical properties (Putthanarat et al., 2004).

A carbodiimide-based coupling strategy was employed to couple NeutrAvidin with silk fibroin in solution or with silk microspheres. NeutrAvidin coupled to silk fibroin solution or silk microspheres retained the ability to bind biotin, including fluorescently labeled biotin (Atto 610), biotinylated-HRP and biotinylated anti-CD3 antibody (the latter functionalization resulted in specific binding of the functionalized silk microspheres to CD3-positive T-lymphocytic cells) (Wang *et al.*, 2011). Insulin was covalently coupled to silk fibroin powder using gluteraldehyde and the resulting insulin-fibroin conjugate showed much higher recovery (about 70%) and physicochemical and biological stability insulin coupled to bovine serum albumin (BSA) or unmodified insulin. In vitro plasma half-lives of insulin alone, BSA-insulin, and fibroin-insulin were 16, 20, and 34 h, respectively. The pharmacological activity of the SF-Ins bioconjugates in diabetic rats evidently was about 3.5 times as long as that of the native insulin (Zhang *et al.*, 2006).

Unlike other gelation processes, which require harsh conditions to crosslink polymer chains, sonication-induced silk hydrogels form physical crosslinks without exposing the incorporated therapeutic to stresses such as shear, temperature or organic solvents (Wang *et al.*, 2008b). As such, silk hydrogels are an attractive immobilization matrix for fragile proteins. NeutrAvidin decorated silk fibroin retained its ability to self-assemble into hydrogel after reaction (Wang *et al.*, 2011). Guziewicz *et al.* loaded monoclonal antibodies into sonicated silk and lyophilized the antibody loaded silk hydrogels to further improve long-term storage stability. The bioactivity of the antibody released from the silk lyophilized gels was confirmed in a cell-based bio-assay (Guziewicz *et al.*, 2011).

4. Small Molecule Stabilization by Silk

Stabilization of small molecules presents different challenges than protein stabilization. Unlike proteins, which can lose bioactivity when structural loss occurs, small molecule degradation predominantly occurs via chemical degradation. However, the unique physical

properties of silk can also improve the stability of small molecules, including dyes and pigments, antioxidants, antibiotics and chemotherapeutics (Table 4).

Though relatively stable in solution (Kaltenbach *et al.*, 2011), the small molecule anticonvulsant adenosine is cleared rapidly and has a short half-life when injected. However, sustained *in vivo* effects were observed when adenosine was encapsulated in silk microspheres suspended in silk sponges and implanted in the hippocampi of kindled epileptic rats. A dose dependent delay in seizure acquisition was observed (Wilz *et al.*, 2008) and implants designed to release 1000 ng adenosine a day completely protected kindled rats from seizures over a 10-day period (Szybala *et al.*, 2009). These studies demonstrated that encapsulation in silk drug carriers represents a safe and effective strategy to sustain adenosine activity at the site of intended action, counteracting rapid local clearance.

Natural pigments such as chlorophyll or carotenoids are attractive due to their safety and biocompatibility, but are less widely used than their synthetic counterparts due to their extreme light-instability once extracted from living bodies. Pigment-fibroin conjugates prepared by adsorbing chlorophyll *a*, β-carotene and astaxanthin onto silk fibroin exhibited high stability to visible and UV light, while fibroin-free pigments were rapidly decolorized by UV light irradiation. The authors attribute this improvement in pigment stability to fibroin's ability to substitute for the proteins that naturally stabilize these materials *in vivo* (Ishii *et al.*, 1995). Immobilization of the oleuropein and rutin from crude olive leaf extract onto silk fibroin was investigated both to purify olive leaf antioxidants and to create an edible, safe, stable of protein-bioactive phytochemical conjugate with antioxidative, antimicrobial, and anti-inflammatory properties. After adsorption of olive leaf antioxidants, the functionalized silk fibroin exhibited antioxidative and antimicrobial activity. Further, the antioxidant activity of desorbed polyphenols purified from the extract using silk was nearly 2 times higher than that of the olive leaf extract containing the same amount of desorbed polyphenols (Bayçin et al., 2010).

Choi *et al*. immobilized two antibiotics (doxycycline and ciprofloxacin) onto silk fibers using a range of conditions and found that doxycycline and ciprofloxacin "dyed" silk fibers were able to inhibit local growth of *Staphylococcus epidermis* lawns on agar plates for at least 48 or 24 hours, respectively (Choi *et al.*, 2004). Encapsulation in silk preserved curcumin's bioactivity, and improved intracellular uptake and drug efficacy; curcumin packaged into silk nanoparticles (<100 nm) reduced breast cancer viability in vitro (Gupta *et al.*, 2009). Recently, the capacity of silk carriers to sequester, stabilize and release bioactive antibiotics was studied (Pritchard et al., in preparation). Incorporation of penicillin and tetracycline into silk films dramatically improved stability compared with storage in solution, and also improved stability compared with storage as dry powder. Stabilization and protection persisted above physiological temperatures (e.g., up to 60°C) and for extended time frames (e.g., up to 6 to 9 months). Preservation of bioactivity during carrier fabrication and drug release was also confirmed for sponges loaded with rifampicin and erythromycin. The latter, though highly unstable in aqueous media (Briseart *et al.*, 1996), continued to inhibit *S. aureus* growth up to 35 days despite being incubated at 37°C for over a month in a hydrated silk sponge (Pritchard *et al.*, in preparation). Tetracycline bioactivity was also

preserved during fabrication of silk microneedle arrays and subsequent in vitro release studies in *Staphylococcus aureus* lawns (Tsioris *et al.*, 2011).

Because silk film encapsulation and/or immobilization stabilized incorporated compound function, doping strategies can be built into silk optical devices to produce multi-functional, biodegradable devices for applications like diagnostics and biosensors. Silk films patterned with micro-prism arrays (MPA) were shown to provide enhanced optical signal through tissues and could be functionalized with inclusion of the chemotherapeutic drug doxorubicin for combined therapy and monitoring. Loading of the doxorubicin into silk MPAs dramatically enhanced storage stability compared with storage in solution: after 3 weeks stored at -20°C (frozen) and 60°C, the fluorescence of the doxorubicin stored in silk films did not significantly decrease at either storage temperature, while less than 80% and 30% of fluorescent doxorubicin remained for storage in solution at -20°C and 60°C, respectively (Tao *et al.*, submitted). Lawrence *et. al* integrated biological substances into specialized optical elements prepared by casting aqueous silk solution and analyzed the functionality of the incorporated substances within the solidified silk films. By casting on diffractive molds, optical properties of the patterned silk film and biological function of the dopant could be consolidated into a single multi-functional biomaterial device. When the small molecule organic pH indictor phenolsulfonphthlein (Phenol Red) was incorporated into a patterned silk grating, both the optical function and functionality of the small molecule dopant were retained. The optical function of the diffraction grating was confirmed by showing propagating supercontinuum radiation onto the grating, while the pH sensing capability of the incorporated Phenol Red was shown through variations of the spectrum correlated to the acidty of the dip solution when gratings were immersed into solutions of different pH (Lawrence *et al.*, 2008). Similarly, silk protein was chemically modified with 4-aminobenzoic acid to perform pH sensing in active optofluidic devices. The modified silk was found to be stable and spectrally responsive over a wide pH range (Tsioris *et al.*, 2010)

5. Proposed Mechanisms of Silk-Based Stabilization

There are many potential sources of protein or small molecule instability, and silk may exert a combination of different stabilizing effects. As described earlier, preservation of bioactivity during silk device fabrication may be attributed to the use of mild, aqueous processing options made possible due to the unique chemistry, structure and assembly of silk into nanodomains with reduced water content (Figure 1). The following mechanisms (or, more likely, a combination of the following mechanisms) have been proposed to explain the stabilization effects of immobilization or conjugation with silk fibroin during storage and duration *in vitro* or *in vivo* release or exposure (silk stabilization effects are also summarized in Figure 1):

Protective Barrier Effects

At their intended site of action, proteins and small molecules may be subject to enzymatic clearance and degradation, for example the proteolytic enzymes encountered in the GI tract during oral administration or the antibiotic-degrading β-lactamase enzymes released by methicillin-resistant *Staphylococcus aureus* (MRSA) (Turos *et al.*, 2007). Once immobilized in silk carriers, the susceptibility of small molecules and proteins to enzymatic degradation

decreases dramatically due to their reduced accessibility to exogenous enzymes. Protective effects may also result from the silk protein binding inactivators, as has been described for high concentrations of hemoglobin or albumin. Immobilization also protects most of the incorporated small molecules or protein from the external interface, reducing air-water interface or oil-water interface related instability.

Reduced Mobility Effects

Dehydration (i.e. via lyophilization or small molecule storage in a dry powder state) is believed to stabilize small molecules and proteins by reducing molecular mobility. Immobilization in or on a solid silk matrix, particularly when the drug carrier is stored in a dehydrated state, has a comparable stabilization effect on encapsulants, especially proteins. Aggregation is a common source of protein inactivation. Small molecules may also form aggregates that fall out of solution, thereby losing activity. When proteins or small molecules are dispersed in or attached to silk matrices, mutual spatial fixation and reduction of molecular mobility prevent aggregation. Another major source of protein activity loss (particularly in response to heating, pH shifts and denaturing agents like organic solvents) is unfolding, i.e. conformational change of the protein's structure. Immobilization (either through entrapment, adsorption or chemical coupling to the silk biomaterial surface) would be expected to increase protein rigidity and reduce conformational mobility. This "freezing" of the protein in its optimal, native structure and reduced molecular mobility reduces protein unfolding compared to free proteins, thereby improving thermal stability. This explanation also describes reduced dissociation of immobilized multimeric proteins, particularly when proteins are entrapped; attachment to polymer surfaces will only reduce dissociation is all the subunits are attached. Hydrogen bond formation between imbedded proteins and the silk fibroin protein may further reduce mobility and promote protein stability.

Microenvironment Effects

Encapsulation in polymer matrices (particularly silk due to its unique structure) produces protective microenvironments. Embedded proteins and small molecules sequestered in these microenvironments are expected to experience a degree of buffering from changes that occur in the bulk solution. Improved storage, more stable conditions in fibroin matrix microenvironments may contribute to increased stabilization against pH inactivation, organic solvents and hydrogen peroxide. Further, the dominating hydrophobic nature of silk proteins excludes water from the crystallized beta sheet domains, while also limiting water content in the less crystalline yet still hydrogen bonded spacers or more hydrophilic regions. Since these structural features remain nanoscale in size, water content available to denature or resolubilize entrapped compounds is limited.

Table 5 summarizes the types of silk-based stabilization investigated by authors covered in this review. Processing stability refers to any demonstration that bioactivity or structure of the incorporated compound or protein are preserved during the fabrication of the carrier (i.e., during bulk-loading into films or sponges, encapsulation in micro- or nano-particles, chemical coupling or crosslinking or adsorption). Storage stability designates studies that investigated stabilization during simulated storage. Enhanced thermostability is categorized as storage stability even at temperatures above intended storage conditions, as it provides

accelerated degradation information. Operational stability refers to retention of activity during use of the immobilized protein or small molecule. For immobilized enzymes or biosensors, operational stability can include evidence of stability during repeated industrial processing cycles or diagnostic tests. For therapeutic compounds or proteins (silk drug carriers or scaffolds), sustained release or increased duration of and in vitro or in vivo effect compared with administration of soluble protein or small molecule is categorized as operational stability. Enhanced resistance to proteolysis is also categorized as operational stability, as proteases are unlikely to be encountered during long-term storage. Enhanced pH stability is classified as operational stability and enhanced light stability is classified as storage stability. A plus sign (+) indicates that the study provided evidence of stabilization effects in this category. A minus sign (-) indicates that the study did not provide evidence of stabilization in this category, not that the study provided evidence that stabilization effects did not occur in this category. For example, if bioactivity of a protein-loaded silk scaffold is demonstrated in vitro or in vitro with no mention of a storage period post-fabrication, the study is considered to have included evidence of processing stability and operational stability (bioactivity was maintained during fabrication, and retained during in vitro culture of cells or in vivo implantation), but not storage stability.

6. Conclusions

In addition to its ability to support cell growth as a tissue engineering scaffold and to sustain and control release as a drug carrier for controlled-release implants, silk fibroin possesses a unique ability to stabilize immobilized proteins (especially enzymes and growth factors) and small molecules. Silk immobilization via covalently coupling, adsorption, entrapment or encapsulation enhances the stability of small molecule and proteins against heat, pH and proteolysis. The use of mild, aqueous processing options stabilizes during silk carrier fabrication, while a combination of protective barrier, microenvironment and reduced compound or protein mobility enhance stabilization during storage and exposure to in vitro, in vivo or operational conditions. Given the exceptional capacity of silk to stabilize sensitive incorporated compounds, we anticipate silk biomaterials will be attractive carriers for gene delivery, vaccines and volatile flavors and fragrances.

Acknowledgments

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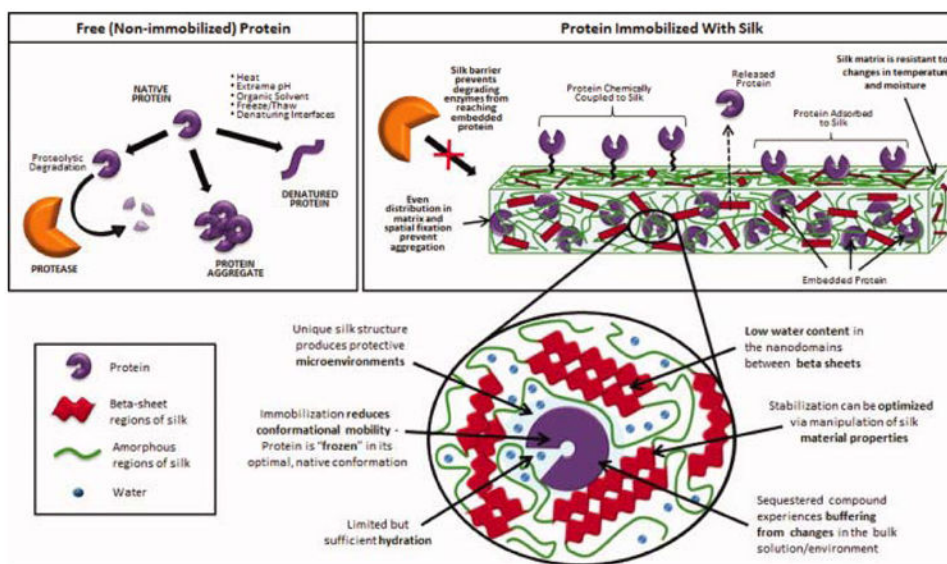Zhu G, Mallery SR, Schwendeman SP. Nat Biotechnol. 2000; 18:52–57. [PubMed: 10625391]

Figure 1. Physical and chemical aspects of stabilization of compounds in silk

Table 1

Silk Stabilization of Enzymes

Enzyme Immobilized	Silk Immobilization Approach	Key Stabilization Results	Reference
Horseradish peroxidase (HRP)	Bulk loaded silk films (silk and HRP solutions mixed, cast and dried; films rendered water insoluble with 75% ethanol)	• Response of hydrogen peroxide sensors based on HRP immobilized in silk films only decreased 16% after 2 months of storage at 4°C	Qian *et al.*, 1996
	Bulk loaded silk films (silk and HRP solutions mixed, cast and dried; films rendered water insoluble with 75% ethanol)	• Eighty percent relative activity was maintained after 45 days' storage at 4°C	Qian *et al.*, 1997
	Bulk loaded silk films (silk and HRP solutions mixed, cast and dried; films rendered water insoluble with 90% methanol)	• Enzymatically active HRP released over 4 weeks	Hofmann *et al.*, 2006
	HRP gradients immobilized within three-dimensional silk fibroin sponges using either chemical coupling or adsorption	• After a 30 min incubation at 60°C, free enzyme lost all activity; compared to approx. 75% and 25% activity remaining for coupled and adsorbed HRP, respectively • After 30 min incubation at 37°C, activity of HRP covalently coupled to silk sponges increased above 100% • Adsorption-loaded HRP gradient preserved during incubation for 4 days in PBS solution	Vepari and Kaplan, 2006
	Bulk loaded silk microparticles (lipid template encapsulation)	• Enzymatically active HRP released over 10-15 days	Wang *et al.*, 2007
	Bulk loaded silk films (silk and HRP solutions mixed, cast on nanopatterned PDMS molds, dried; films rendered water insoluble by water annealing)	• Enzymatic activity of incorporated HRP confirmed	Lawrence *et al.*, 2008
	Bulk loaded silk films (silk and HRP mixed, case and dried; rendered water insoluble with 90% methanol)	• After 5 months of storage, HRP immobilized in silk films retained 24%, 22%, 17% of the initial activity when stored at 4°C, room temperature and 37°C, respectively • Enzyme stability higher in untreated films than methanol treated films • Enzyme stability higher in films with higher HRP loading compared with lower loading	Lu *et al.*, 2009
	Bulk loaded silk films (silk and HRP solutions mixed with or without 30% glycerol, cast and dried; films rendered water with water annealing, methanol or stretching)	• After 2 months of storage, HRP immobilized in silk film retained more than 90% of the initial activity at 4°C, room temperature and 37°C	Lu *et al.*, 2010

Enzyme Immobilized	Silk Immobilization Approach	Key Stabilization Results	Reference
		• Unique periodic hydrophobic-hydrophilic domains of silk's structure has a stabilizing effect on incorporated proteins	
	Bulk loaded silk films (silk and HRP solutions mixed, cast on microneedle array PDMS molds, dried; films rendered insoluble by water annealing or methanol)	• Enzymatic activity of incorporated HRP confirmed • Enzyme release from microneedles sustained up to 48 hours in a collagen hydrogel transdermal model	Tsioris *et al.*, 2011
Glucose oxidase (GOx)	Bulk loaded silk films (silk and GOx solutions mixed, cast and dried; films rendered water insoluble with 80% methanol or gluteraldehyde treatment)	• Activity unchanged after heating at 60°C for 20 min • Activity retained at least 1 month • Stability of GOx stored in silk films improved compared with gelatin films (gelatin films were unstable at elevated temperatures)	Kuzuhara *et al.*, 1987
	Bulk loaded silk films (silk and GOx solutions mixed, cast and dried; films rendered water insoluble with stretching)	• Enzyme activity and stability dependent on silk film characteristics (beta sheet content and matrix permeability) • Immobilization improved thermal and pH stability: minimal activity loss for immobilized enzyme at 50°C, compared to 70% activity loss for free enzymes	Demura and Asakura, 1989
	Bulk loaded silk films (silk and GOx solutions mixed, cast and dried; films rendered water insoluble with physical treatments: stretching, compressing and standing under high humidity and methanol-immersion treatment)	• Improvement in the pH and thermal stability of the immobilized enzyme compared with free enzyme • GOx immobilized in fibroin films retained more than 90% relative activity after soaking in phosphate buffer at pH 7 at 4°C for 4 months • At 50°C, free enzyme loses 30% activity, while the relative activity of the immobilized enzyme scarcely changed	Demura *et al.*, 1989
	Bulk loaded porous silk membrane (silk, GOx and PEG mixed, cast and dried)	• Membrane permeability to glucose and NaCl increase with increasing PEG concentration • Improved glucose permeability/sensor functionality compared to earlier non-porous membranes	Demura and Asakura, 1991
	Bulk loaded silk films (silk and GOx solutions mixed, cast and dried; method	• Glucose detecting ability was retained for over 2 years in films stored at 4°C	Zhang *et al.*, 1998

Enzyme Immobilized	Silk Immobilization Approach	Key Stabilization Results	Reference
	of rendering water insoluble not reported)	• Enzymatic activity was also maintained when GOx loaded silk films were immersed in buffers with varied pH (range of 5.0-10.0), exposed to temperatures from 20-50°C and used repeatedly for over 1,000 detections	
	GOx bulk loaded in silk films and blended silk-poly(vinyl alcohol) films	• Immobilization improved pH stability and thermal stability compared with free GOx and GOx immobilized via glutaraldehyde crosslinking onto carbon electrodes • After 30 min incubation at 50°C, enzyme immobilized in silk membranes retained 93% activity, enzyme immobilized in silk-PVA blend films retained 86% activity and free enzyme activity dropped to 30%	Liu *et al.*, 1996
	Bulk loaded silk films (silk and HRP mixed, cast and dried; rendered water insoluble with 90% methanol)	• Untreated films loaded with 1 wt % GOx stored at 4°C and 25°C exhibited slight activity loses during the first 3 months of storage, then during the remaining five months of storage • GOx activity increased above 100% of the initial activity GOx stability was higher in untreated films compared with methanol treated films	Lu et al., 2009
Lipase	Enzyme immobilized on silk fibers via cross-linking with glutaraldehyde	• Immobilized enzyme stable for 2 months at 4°C • At room temperature, activity of both the immobilized and free lipase declined, but relative stability of the immobilized lipase was significantly higher than the free lipase	Chatterjee *et al*., 2009
	Bulk loaded silk films (silk and HRP mixed, case and dried; rendered water insoluble with 90% methanol)	• Significant activity retention for enzymes entrapped in silk films over 10 months even at 37°C • Correlation observed between silk protein processing and enzyme stability suggests that stability could be optimized via processing	Lu et al., 2009
	Enzyme adsorbed onto silk woven fabric: either PDMS-treated fiber (hydrophobic) or native fiber (hydrophilic)	• 2 fold increase in activity on a hydrophobic support compared to a hydrophilic support • Immobilization enhances pH and temperature stability	Chen *et al*., 2010

Enzyme Immobilized	Silk Immobilization Approach	Key Stabilization Results	Reference
Cholesterol oxidase (ChOx)	Chemically coupled onto fibrous, porous woven silk mats	• Half-life(time at which 50% of initial activity remained) of the immobilized ChOx stored in a closed container at 4°C = nearly 13 months	Saxena *et al.*, 2010
Tyrosinase	Bulk loaded silk films (silk and enzyme solutions mixed, cast and dried; films rendered water insoluble with gluteraldehyde treatment)	• Immobilization improved tyrosinase stability over 10 days of storage • Enzyme retained 80% of original activity after repeated reuses	Acharya *et al.*, 2008
Ribonuclease	Chemically coupled to woven silk substrates	• Enzyme activity increased with increased immobilization time • Protein structure and enzymatic activity maintained during immobilization	Cordier *et al.*, 1982
Alkaline phosphatase (ALP)	Immobilized on woven silk substrates by chemical or adsorption methods	• Preparations of alkaline phosphatase retained bioactivity following coupling to silk substrate	Grasset *et al.*, 1983
β-glucosidase	Bulk loaded silk films (silk and enzyme solutions mixed, cast and dried; films rendered water insoluble with 50% ethanol)	• Stability of immobilized enzyme compared with free enzyme substantially enhanced against heating, electrodialysis and protease • Activity after 30 min incubation at 55°C = 50% for soluble enzyme, 90% for immobilized enzyme • Exposure to papain reduced activity of soluble enzyme, but did not affect immobilized enzyme	Miyairi *et al.*, 1978
Uricase	Bulk loaded silk films (silk and uricase solutions mixed, cast and dried; films rendered water insoluble with 80% methanol)	• Biosensor based on immobilized uricase in silk film showed high recoveries of uric acid from serum and urine (>92%) • Immobilized enzymes stable in dry state for over 2 years at 4°C, stable in buffer for 3-4 months • Enzyme activity loss after >1000 measurements was only 10%	Zhang *et al.*, 1998
Heme proteins (myoglobin, hemoglobin, horseradish peroxidase, and catalase)	Bulk loaded silk films (silk and protein mixture cast on the surface of a graphite electrode, dried, rendered insoluble with 80% methanol)	• Heme-proteins maintained their native state in the silk films • Stable in buffer for 12 days • Only a 10% decrease in peak current after storage at 4°C for 1 month	Wu *et al.*, 2006

Enzyme Immobilized	Silk Immobilization Approach	Key Stabilization Results	Reference
L-asparaginase (ASNase)	Enzyme coupled covalently to silk fibroin powder with glutaraldehyde	• Silk conjugated enzyme exhibited enhanced thermostability, pH stability, and resistance to trypsin digestion • Increased storage stability: remaining activity after 30 days at room temperature = over 80% for silk-conjugated ASNase, 30% for unmodified ASNase	Zhang *et al.*, 2005
Phenylalanine ammonia-lyase (PAL)	Bulk loaded silk microparticles (silk solution and enzyme solution mixed, salted out and filtered) (particle diameter <150 μm)	• Increased storage stability: remaining activity after 82 days at 4°C = 75.4% for silk-entrapped PAL, 34.4% for lyophilized free PAL • Encapsulation increased resistance to proteolysis by chymotrypsin and trypsin in vitro • Retention of bioactivity in vivo following oral administration	Inoue *et al.*, 1986
Invertase	Bulk loaded silk fibroin powder (silk and enzyme hydrogel lyophilized, then ground to powder)	• Thermal stability increased for immobilized enzyme compared with free enzyme	Yoshimizu and Asakura, 1990
Organophosphorus Hydrolase (OPH)	Bulk loaded silk films (silk and OPH mixed, cast and dried; films rendered water insoluble with 30% ammonium sulfate)	• Increased enzyme stability at high temperatures • Increased enzyme stability against UV • Resistance to detergent and organic solvents	Dennis, *et al.* in preparation

Table 2

Silk Stabilization of Growth Factors

Growth Factor	Silk Scaffold Immobilization Approach	Bioactivity Results	Reference
BMP-2	Adsorption or covalent coupling to silk films	• Increase in osteogenesis for hMSCs grown on BMP-2 decorated silk films compared with hMSCs grown on control films or exposed to soluble BMP-2	Karageorgiou *et al*., 2004
	Adsorption onto 3D porous aqueous-derived silk sponge	• Enhanced mineralization at the site of highest BMP-2 retention (center of silk sponges)	Karageorgiou *et al*., 2006
	Bulk loaded in electrospun silk nanofiber mats	• Increased osteogenesis of hMSCs cultured for 31 days on BMP-2-loaded scaffolds compared with empty control scaffolds	Li *et al*., 2006
	Adsorption onto 3D porous HFIP-derived silk sponge	• Pre-loading of silk sponges with BMP-2 enhanced in vivo bone formation compared with control silk sponges in a murine mid-femoral segmental defects	Kirker-Head *et al*., 2007
	Encapsulation in silk microspheres (prepared by ethanol precipitation method)	• In vitro BMP-2 silk microspheres induced greater osteogenesis (increased alkaline phosphatase activity and mineralization) in C2C12 cells than soluble growth factor • Injected BMP-2 loaded mircospheres promoted bone formation in vivo in a rat ectopic bone model	Bessa *et al*., 2010
	Bulk loaded silk hydrogels (gelation induced via sonication of aqueous silk solution)	• Injected BMP-2 loaded silk hydrogels promoted bone formation in vivo using a critically-sized rat femoral segmental defect model	Diab *et al*., 2011
FGF-2	Adsorption onto silk films (retention enhanced by chemical decoration of fibroin with sulfonic acid moieties)	• hMSCs cultured on sulfonated silk films with adsorbed FGF-2 exhibited reduced metabolic activity and increased levels of pERK1/2	Wenk *et al*., 2010
IGF-I	Bulk loaded porous 3D scaffolds (prepared by adding growth factors and silk mixture to porous molds, freeze-drying and immersing in 90% methanol)	• hMSCs cultured on IGF-I loaded scaffolds exhibit enhanced chondrogenic differentiation compared with hMSCs cultured on unloaded control silk scaffolds • Growth factor potency retained during methanol treatment of the scaffolds	Uebersax *et al*., 2008
	Encapsulation in silk microspheres (prepared by laminar jet break-up of aqueous solutions, followed by water vapor annealing)	• Enhanced in vitro proliferation of MG-63 cells compared with controls	Wenk *et al*., 2008
PTH	Chemical coupling to silk films	• Increased adhesion of osteoblast-like cells on RGD and PTH decorated silk films compared to plastic or control peptide	Sofia *et al*., 2001
bFGF	Adsorption onto 3D porous silk sponges (aqueous- or HFIP-derived)	• Bioactivity not reported • Scaffolds sustained bFGF release at implantation site up to 2 weeks (compared to rapid dissipation of soluble injected bFGF within one day)	Wongpanit *et al*., 2010
NGF	Bulk loaded porous tube-shaped scaffolds (prepared by freeze	• NGF loaded scaffolds supported PC12 cell differentiation with neurite outgrowth	Uebersax *et al*., 2007

Growth Factor	Silk Scaffold Immobilization Approach	Bioactivity Results	Reference
	drying molds filled with aqueous solution of NGF and silk)	• Growth factor potency retained during methanol treatment of the scaffolds	
GDNF NGF	Bulk loaded silk film tubes	• Various neuronal cells from chicken embryos (DRG (dorsal root ganglions) sensory neurons and spinal cord (SpC) motor neurons) cultured on growth factor loaded silk guidance tubes exhibited augmented length and rate of axonal outgrowth	Madduri *et al.*, 2010
BMP-2 IGF-I	Lipid template silk fibroin microspheres incorporated into aqueous-derived silk porous scaffolds using a gradient process	• Both growth factors formed deep and linear concentration gradients in the scaffolds that induced and controlled hMSC differentiation	Wang *et al.*, 2009
BMP-2 VEGF	Bulk loaded silk hydrogels (gelation induced via sonication of aqueous silk solution)	• Injected GF loaded silk hydrogels promoted angiogenesis and new bone formation in vivo in a rabbit maxillary sinus floor augmentation model • Additive effect observed for BMP-2 in combination with VEGF	Zhang *et al.*, 2011

Abbreviations: BMP-2, Bone morphogenetic protein 2; FGF-2, Fibroblast growth factor 2; IGF-I, Insulin growth factor I; PTH, Parathyroid hormone; bFGF, Basic fibroblast growth factor; NGF, Nerve growth factor; GDNF, Glial cell line-derived neurotrophic factor; VEGF, Vascular endothelial growth factor; pERK1/2, Phosphorylated extracellular signal-regulated kinases; hMSC, Human mesenchymal stem cells; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol;

Table 3

Silk Stabilization of Proteins

Protein Immobilized	Silk Immobilization Approach	Key Stabilization Results	Reference
Staphylococcal low molecular weight Protein A (LPA)	Bulk loaded silk films (silk and protein solutions mixed, cast, dried; films rendered insoluble by water annealing)	• Immobilization increased LPA thermal stability relative to free LPA (based on immunoglobulin G binding) • At 120°C, immobilized LPS retained 50% of its relative IgG binding compared to less than 20% for free LPA	Kikuchi *et al.*, 1999
Hemoglobin	Bulk loaded silk films (silk and protein solutions mixed, cast on nanopatterned PDMS molds, dried; films rendered insoluble by water annealing)	• Oxygen binding functionality of hemoglobin entrapped in silk confirmed with an optical transmission experiment	Lawrence et al., 2008
Normal murine IgG	Immobilization and enrichment at the silk film surface via addition of antibody solution to the surface of a concentrated, partially dried, semi-solid silk film, followed by drying	• Immobilized antibody stored at 25°C in dry conditions for 4 weeks retain approx. 40% of its secondary antibody binding activity	Lu *et al.*, 2011
Green Fluorescent Protein (GFP)	Bulk loaded silk films (silk extracted from silkworm glands and diluted, silk and protein solutions mixed, cast and annealed at 20°C)	• GFP molecules immobilized in silk films maintain their nonlinear optical properties	Putthanarat *et al.*, 2004
NeutrAvidin	Chemically coupled to silk microspheres and to silk protein in solution prior to hydrogel formation	• Silk-coupled NeutrAvidin retained biotin-binding ability • NeutrAvidin decorated silk microspheres coupled to biotinylated anti-CD3 antibody exhibited specific targeting of CD3-positive T-lymphocytic cells	Wang *et al.*, 2011
Insulin	Covalently coupled to silk fibroin powder using gluteraldehyde	• Insulin-fibroin conjugate showed higher recovery (about 70%) and higher physicochemical and biological stability than insulin coupled to bovine serum albumin (BSA) or unmodified insulin • Evident pharmacological activity of the SF-Ins bioconjugates in diabetic rats was ~3.5 times as long as that of the native insulin	Zhang *et al.*, 2006
Murine anti-TGFb IgG1 monoclonal antibody	Bulk loaded silk hydrogels (gelation induced by sonication)	• Bioactivity of the antibody released from the silk lyophilized gels confirmed in a cell-based bio-assay	Guziewicz *et al.*, 2011

Table 4

Silk Stabilization of Small Molecules

Small Molecule Immobilized	Silk Immobilization Approach	Key Stabilization Results	Reference
Adenosine	Drug encapsulated into silk microspheres, loaded into silk sponges and coated with drug-loaded silk films	• Duration of sustained in vitro adenosine release corresponded to duration of in vivo therapeutic effect (seizure suppression in a kindled rat model of epilepsy) • Bioactivity of adenosine encapsulated in silk preserved in vivo up to 10 days	Wilz et al., 2008; Szybala et al., 2009
Chlorophyll *a* β-carotene Astaxanthin	Adsorption onto silk powder	• Silk-pigment conjugates exhibited high UV light stability, while free pigments decolorized rapidly when exposed to UV light	Ishii et al., 1995
Antioxidants from crude olive leaf extract (oleuropein and rutin)	Adsorption onto silk powder	• Functionalized silk fibroin exhibited antioxidative and antimicrobial activity • Antioxidant activity of desorbed polyphenols following adsorption to silk nearly doubled compared to crude extract	Bayçin et al., 2010
Doxycycline Ciprofloxacin	Adsorption onto silk fibers	• Functionalized silk fibers repressed local bacterial lawn growth for at least 48 hours (doxycycline) or 24 hours (ciprofloxacin)	Choi et al., 2004
Curcumin	Encapsulated in silk nanoparticles	• Bioactivity preserved during encapsulation • Curcumin in silk nanoparticles reduced viability of breast cancer cells in vitro	Gupta *et al.*, 2009
Various antibiotics (penicillin, tetracycline, rifampicin, erythromycin)	Various immobilization approaches, including bulk loading, adsorption and encapsulation	• Storage of penicillin and tetracycline in bulk-loaded silk films improved stability compared to storage in solution or as dry powder • Stabilization persisted above physiological temperatures (e.g. up to 60°C) at least 6 months • Bioactivity of erythromycin sustained over a month in hydrated sponge	Pritchard *et al.*, in preparation
Tetracycline	Bulk loaded silk films (silk and antibiotic solutions mixed, cast on microneedle array PDMS molds, dried; films rendered insoluble by water annealing or methanol)	• Bioactivity of incorporated tetracycline confirmed based on growth inhibition of *S. aureus* lawns	Tsioris *et al.*, 2011

Small Molecule Immobilized	Silk Immobilization Approach	Key Stabilization Results	Reference
Doxorubicin	Bulk loaded silk films (silk and antibiotic solutions mixed, cast on microprism array (MPA) PDMS molds, dried; films rendered insoluble by water annealing)	• After 3 weeks stored at -20°C (frozen) and 60°C, fluorescence of the doxorubicin stored in silk films did not significantly decrease, compared to less than 80% and 30% of fluorescent doxorubicin remaining when stored in solution at -20°C and 60°C, respectively	Tao *et al.*, submitted
Phenol-sulfon-phthlein (Phenol Red)	Bulk loaded silk films (silk and HRP solutions mixed, cast on nanopatterned PDMS molds, dried; films rendered insoluble by water annealing)	• Functionality (i.e. pH sensing capability) of incorporated indicator demonstrated by immersing Phenol Red-loaded films in solutions of varying acidity and observing resulting spectrum variations	Lawrence *et al.*, 2008
4-amino-benzoic acid	Chemically coupled to silk film	• Functionality (i.e. pH sensing capability) and stability of the silk-coupled indicator demonstrated over a wide pH range	Tsioris *et al.*, 2010

Table 5

Summary of Silk Stabilization Effects: Processing, Storage and Operational Stability

Protein or Compound Immobilized	Silk Material Format	Stabilization Effects			Ref.
		Processing Stability	Storage Stability	Operational Stability	
Enzymes – Horseradish Peroxidase (HRP)					
Horseradish peroxidase (HRP)	Bulk loaded silk films	+	+	-	Qian et al., 1996
	Bulk loaded silk films	+	+	-	Qian et al., 1997
	Bulk loaded silk films	+	-	+	Hofmann et al., 2006
	Chemical coupling or adsorption onto silk sponges	+	+	+	Vepari and Kaplan, 2006
	Microparticle encapsulation	+	-	+	Wang et al., 2007
	Bulk loaded silk films (nanopatterned)	+	-	-	Lawrence et al., 2008
	Bulk loaded silk films	+	+	-	Lu et al., 2009
	Bulk loaded silk films	+	+	+	Lu et al., 2010
	Bulk loaded silk films (microneedle arrays)	+	-	+	Tsioris et al., 2011
Enzymes – Glucose Oxidase (GOx)					
Glucose oxidase (GOx)	Bulk loaded silk films	+	+	-	Kuzuhara et al., 1987
	Bulk loaded silk films	+	+	+	Demura and Asakura, 1989
	Bulk loaded silk films	+	+	+	Demura et al., 1989
	Bulk loaded porous silk films	+	-	-	Demura and Asakura, 1991
	Bulk loaded silk films	+	+	+	Zhang et al., 1998
	Bulk loaded silk films and blended silk-PVA films	+	+	+	Liu et al., 1996
	Bulk loaded silk films	+	+	-	Lu et al., 2009
Other Enzymes					
Lipase	Glutaraldehyde crosslinking onto silk fibers	+	+	+	Chatterjee et al., 2009
	Bulk loaded silk films	+	+	-	Lu et al., 2009
	Adsorption onto silk woven fabric	+	+	+	Chen et al., 2010
Cholesterol oxidase	Chemical coupling onto woven silk substrates	+	+	-	Saxena et al., 2010
Tyrosinase	Bulk loaded silk films	+	+	+	Acharya et al., 2008

Protein or Compound Immobilized	Silk Material Format	Stabilization Effects			Ref.
		Processing Stability	Storage Stability	Operational Stability	
Ribonuclease	Chemical coupling onto woven silk substrated	+	+	-	Cordier et al., 1982
Alkaline phosphatase	Chemical coupling or adsorption onto woven silk substrates	+	-	-	Grasset et al., 1983
β-glucosidase	Bulk loaded silk films	+	+	+	Miyairi et al., 1978
Uricase	Bulk loaded silk films	+	+	+	Zhang et al., 1998
Heme proteins (myoglobin, hemoglobin, horseradish peroxidase, and catalase)	Bulk loaded silk films	+	+	+	Wu et al., 2006
L-asparaginase (ASNase)	Glutaraldehyde crosslinking to silk powder	+	+	+	Zhang et al., 2005
Phenylalanine ammonia-lyase (PAL)	Encapsulation silk particles	+	+	+	Inoue et al., 1986
Invertase	Bulk loaded silk hydrogel, lyophilized, then ground to powder	+	+	-	Yoshimizu and Asakura, 1990
Organo-phosphorus Hydrolase (OPH)	Bulk loaded silk films	+	+	+	Dennis et al., in preparation
Growth Factors					
BMP-2	Adsorption onto silk films or covalent coupling onto silk films	+	-	+	Karageorgiou et al., 2004
	Adsorption onto 3D porous silk sponge	+	-	+	Karageorgiou et al., 2006
	Bulk loaded in electrospun silk nanofiber mats	+	-	+	Li et al., 2006
	Adsorption onto 3D porous silk sponge	+	-	+	Kirker-Head et al., 2007
	Encapsulation in silk microspheres	+	-	+	Bessa et al., 2010
	Bulk loaded silk hydrogels (gelation induced via sonication of aqueous silk solution)	+	-	+	Diab et al., 2011
FGF-2	Adsorption onto silk films (retention enhanced by chemical decoration of fibroin with sulfonic acid moieties)	+	-	+	Wenk et al., 2010
IGF-I	Bulk loaded porous 3D scaffolds	+	-	+	Uebersax et al., 2008
	Encapsulation in silk fibroin microspheres	+	-	+	Wenk et al., 2008
PTH	Chemical coupling to silk films	+	-	+	Sofia et al., 2001
bFGF	Adsorption onto 3D porous silk sponges (aqueous- or HFIP-derived)	-	-	+	Wongpanit et al., 2010
NGF	Bulk loaded porous tube-shaped scaffolds f	+	-	+	Uebersax et al., 2007
GDNF NGF	Bulk loaded silk film tubes	+	-	+	Madduri et al., 2010

Protein or Compound Immobilized	Silk Material Format	Stabilization Effects			Ref.
		Processing Stability	Storage Stability	Operational Stability	
BMP-2 IGF-I	Lipid template silk fibroin microspheres incorporated into aqueous-derived silk porous scaffolds using a gradient process	+	-	+	Wang et al., 2009
BMP-2 VEGF	Bulk loaded silk hydrogels (gelation induced via sonication of aqueous silk solution)	+	-	+	Zhang et al., 2011
Other Proteins					
Low mol. wt. Protein A (LPA)	Bulk loaded silk films	+	+	+	Kikuchi *et al.*, 1999
Hemoglobin	Bulk loaded silk films	+	-	-	Lawrence et al., 2008
IgG	Immobilization and enrichment on the silk film surface	+	+	-	Lu *et al.*, 2011
Green Fluorescent Protein (GFP)	Bulk loaded silk films	+	-	-	Putthanarat *et al.*, 2004
NeutrAvidin	Chemically coupled to silk microspheres and silk protein in solution	+	-	+	Wang *et al.*, 2011
Insulin	Coupled to silk fibroin powder gluteraldehyde	+	-	+	Zhang et al., 2006
Murine anti-TGFb IgG1 monoclonal antibody	Bulk loaded sonication-induced silk hydrogels	+	-	-	Guziewicz *et al.*, 2011
Small Molecules					
Adenosine	Encapsulated into silk microspheres loaded into silk sponges and coated with drug-loaded silk films	+	-	+	Wilz et al., 2008; Szybala et al., 2009
Chlorophyll *a* β-carotene Astaxanthin	Adsorption onto silk powder	+	+	-	Ishii et al., 1995
Oleuropein Rutin	Adsorption onto silk powder	+	-	+	Bayçin et al., 2010
Doxycycline Ciprofloxacin	Drugs "dyed" onto silk fibers	+	-	+	Choi et al.,
Curcumin	Encapsulated with silk nanoparticles	+	-	+	Gupta *et al.*, 2009
Penicillin, Tetracycline Rifampicin Erythromycin	Bulk-loaded silk films and adsorption	+	+	+	Pritchard *et al.*, in preparation
Tetracycline	Bulk loaded silk films	+	-	+	Tsioris *et al.*, 2011
Doxorubicin	Bulk loaded silk films	+	+	-	Tao *et al.*, submitted
Phenolsulfon-phthlein (Phenol Red)	Bulk loaded silk films	+	-	-	Lawrence *et al.*, 2008
4-amino-benzoic acid	Chemically coupled to silk films	+	-	-	Tsioris *et al.*, 2010