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Engineering chitosan-silk fibroin laminates for use as strong, tough, and biodegradable alternatives to plastic packaging

A thesis presented by
Chelsea Xia

to
The Faculty of the Harvard John A. Paulson School of Engineering and Applied Sciences
and
The Committee on Degrees in Environmental Science and Public Policy
in partial fulfillment of the requirements for
the Bachelor of Arts degree with honors in
Biomedical Engineering & Environmental Sciences and Public Policy

Advisers: Drs. Donald Ingber, Emily Stoler, and Benjamin Freedman

Harvard University
Cambridge, MA
April 1, 2024

Honor Code

In submitting this thesis to the Harvard John A. Paulson School of Engineering and Applied Sciences in partial fulfillment of the requirements for the degree with honors of Bachelor of Arts, I affirm my awareness of the standards of the Harvard College Honor Code.

Name: Chelsea Xia

Signature: *Chelsea Xia*

The Harvard College Honor Code Members of the Harvard College community commit themselves to producing academic work of integrity – that is, work that adheres to the scholarly and intellectual standards of accurate attribution of sources, appropriate collection and use of data, and transparent acknowledgement of the contribution of others to their ideas, discoveries, interpretations, and conclusions. Cheating on exams or problem sets, plagiarizing or misrepresenting the ideas or language of someone else as one's own, falsifying data, or any other instance of academic dishonesty violates the standards of our community, as well as the standards of the wider world of learning and affairs.

List of Contributions

The initial methodology for making chitosan-silk fibroin laminates was devised by Javier Fernandez and Donald Ingber. For this study, experiments were conceptualized and designed with input from my mentor, Emily Stoler. FTIR and Raman spectroscopy were conducted with the help of Arthur McClelland. SEM imaging was conducted by James Weaver. The rest of the experimental data presented here was collected and analyzed by me.

Acknowledgments

There are many people without whom this thesis would not have been possible. Firstly, I am deeply grateful to my mentors, Dr. Emily Stoler and Dr. Benjamin Freedman, for their invaluable guidance throughout this process. Thank you for your scientific expertise, inexhaustible curiosity, and unflagging efforts to make the world a better place—I am inspired every time we speak. I also owe so much to the people who trained me, helped me, and built things with me such that this project never wanted for anything: Dr. Arthur McClelland, Paul Machado, Dr. James Weaver, Dr. Thomas Ferrante, Greg Freeburn, Dr. Nicholas Colella, and Alexander Pauer. Thank you to my advisors in the Biomedical Engineering and Environmental Sciences and Public Policy departments—Dr. Linsey Moyer, Lorraine Maffeo, Dr. Diana Sharpe—for supporting me and giving me the freedom to work on a problem that I truly care about. Thank you to my readers, Dr. Donald Ingber, Dr. David Mooney, and Dr. Peter Huybers, for taking the time to wade through the final product of many months of work, and for their help along the way. And of course, thank you to my friends and family, without whom I might have given up a dozen or so times. You brighten my days and keep me going. I could not have done it without you.

Abstract

Plastics are strong, resilient, and lightweight materials that have become essential to modern life. However, the mass production of plastic has also generated 6.3 billion metric tons of plastic waste, with devastating effects on wildlife, natural ecosystems, and even human health. Thus a need exists for biodegradable plastic substitutes that perform like conventional plastics. This study fabricates, characterizes, and optimizes chitosan-silk fibroin laminate films (termed "Shrilk") for use as biodegradable alternatives to plastic packaging, which makes up 40% of all plastic waste. Shrilk—a portmanteau of the two words, "shrimp" (because chitosan is often isolated from shrimp shells) and "silk" (from silk fibroin)—demonstrated optical clarity and tensile properties comparable to common plastic packaging materials. The laminate structure resulted in higher tensile strength than both pure and blended films. Raman spectroscopy indicated a region of chitosan-silk fibroin overlap at the interface, suggesting that the laminar arrangement enables chitosan and silk fibroin to interact in an organized form of mechanical entanglement. Shrilk films were confirmed to be biodegradable, losing 84% of their total mass after four weeks in simulated landfill conditions. Casein, collagen, and keratin were investigated as potential substitutes for silk fibroin, but were unable to reproduce the adhesion, flexibility, and strength found in Shrilk films. The molecular weight of chitosan, thickness of films, number of laminate layers, and amount of glycerol were varied in order to alter Shrilk's mechanical properties, but generally had little effect. However, covering Shrilk films with a hydrophobic coating effectively prevented Shrilk from completely losing its mechanical properties when wet, and even suggested that a moderate amount of water could act as a plasticizer to increase the films' toughness and elongation at break. Overall, this work presents a strong foundation for future research into chitosan-protein laminates as a potential solution to plastic pollution.

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1. Introduction

1.1 The Plastic Pollution Problem

In 1907, the Belgian chemist Leo Baekeland developed Bakelite, the world's first fully synthetic plastic [1]. Since then, plastics have become a ubiquitous presence in modern life. These strong, durable, and inexpensive materials are used in everything from packaging to clothing to building insulation [2]. In total, an estimated 8.3 billion metric tons of plastics have been produced, with 6.3 billion metric tons eventually becoming plastic waste [3]. Over 350 million tons of plastic waste are generated every year—a number which only continues to grow **(Figure 1.1)** [4]. With only approximately 9% of this waste being recycled and 12% incinerated, 79% of plastic waste is left to build up in landfills or the natural environment [3]. This overwhelming amount of plastic is long-lasting, resistant to decomposition, and causes deleterious effects on environmental, ecological, and human health.

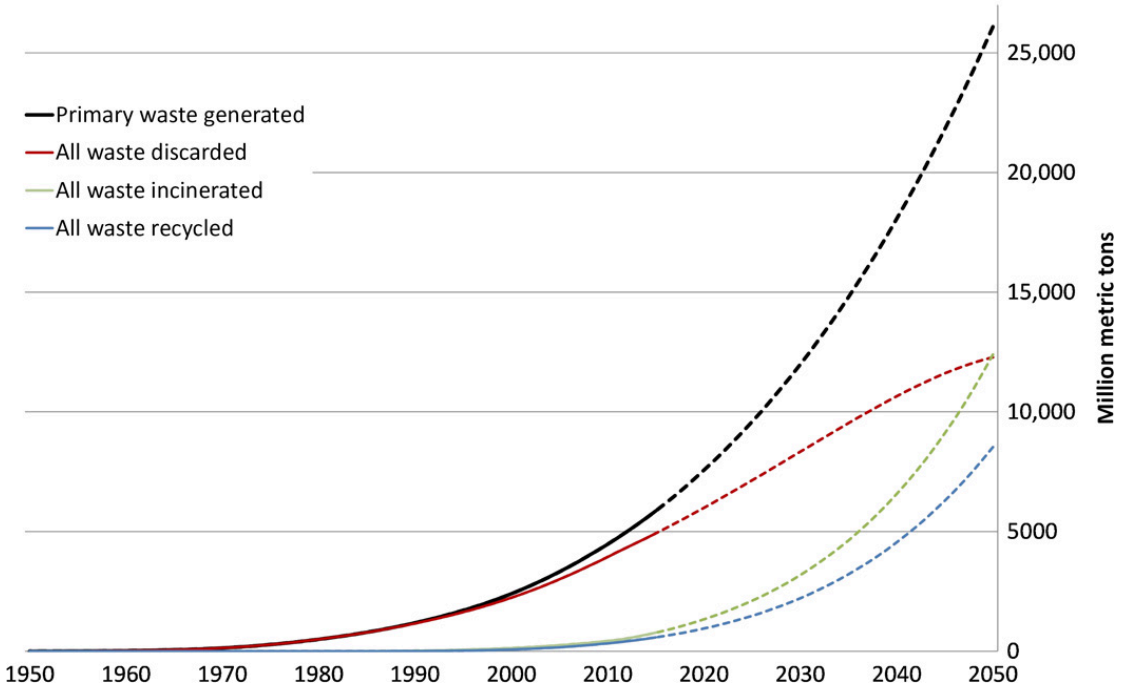


Figure 1.1: Historical and predicted estimates of global waste generation and disposal. Solid lines indicate historical data from 1950-2015. Dashed lines indicate projections up until 2050. Reproduced from Geyer et al. (2017) [3].

In 2022, global revenues for the plastic industry were valued at $598.76 billion, a number which is expected to surpass $827.12 billion by 2030 [5]. However, these revenues come at an enormous environmental cost. In 2019 alone, the plastic production and incineration process produced roughly 850 million metric tons of greenhouse gases [6]. Research suggests that, if plastic production continues to grow according to business-as-usual, the greenhouse gas emissions from plastics will reach 15% of the global carbon budget by 2050 [7]. In the year 2050 alone, the plastic industry is estimated to emit a net 4.5 Gt of carbon dioxide—more than 1% of the global carbon budget recommended by the IPCC in order to have a two-thirds chance of limiting warming to 1.5°C **(Figure 1.2)** [8], [9]. This immense carbon footprint exists due to the fact that over 99% of plastics are derived from fossil fuels. Indeed, the link between plastic production and fossil fuel usage is so strong and entrenched that the plastic and fossil fuel industries are largely dominated by the same companies, such as Dow, DuPont, ExxonMobil, Shell, and Chevron [10].

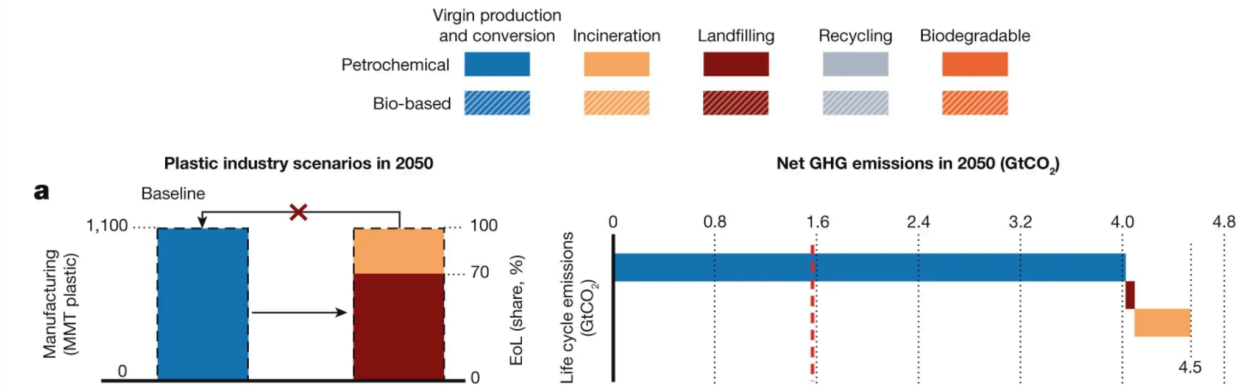


Figure 1.2: Projected greenhouse gas emissions from the plastic industry in 2050. Assumes an estimated global plastic production of 1.1 Gt. The left-hand graph represents the life cycle of plastic, indicating that plastic waste is discarded using a combination of landfill and incineration. The right-hand graph depicts the projected net GHG emissions due to plastic production and disposal. The dashed red line denotes the net GHG emissions of the plastic industry in 2020. Reproduced from the Center for International and Environmental Law (2017) [9].

In addition to their considerable carbon footprint, most commercial plastics do not degrade fully for hundreds of years. While different sources report highly varied environmental lifetimes, a literature review reveals that the mean reported lifetimes for common plastic goods such as plastic water bottles and plastic bags fall squarely within the 100-1000 year range **(Figure 1.3)** [11]. This unmanaged plastic debris accumulates in the ocean, earth, and atmosphere, and has harmful physical and chemical effects on living organisms [12]. Animals often get entangled in plastic waste, increasing the chances of injury, drowning, suffocation, and vulnerability to predation. When ingested, plastic waste causes intestinal bleeding, perforation, and blockage. Ingested plastic can also induce a false sense of satiety, discouraging animals from

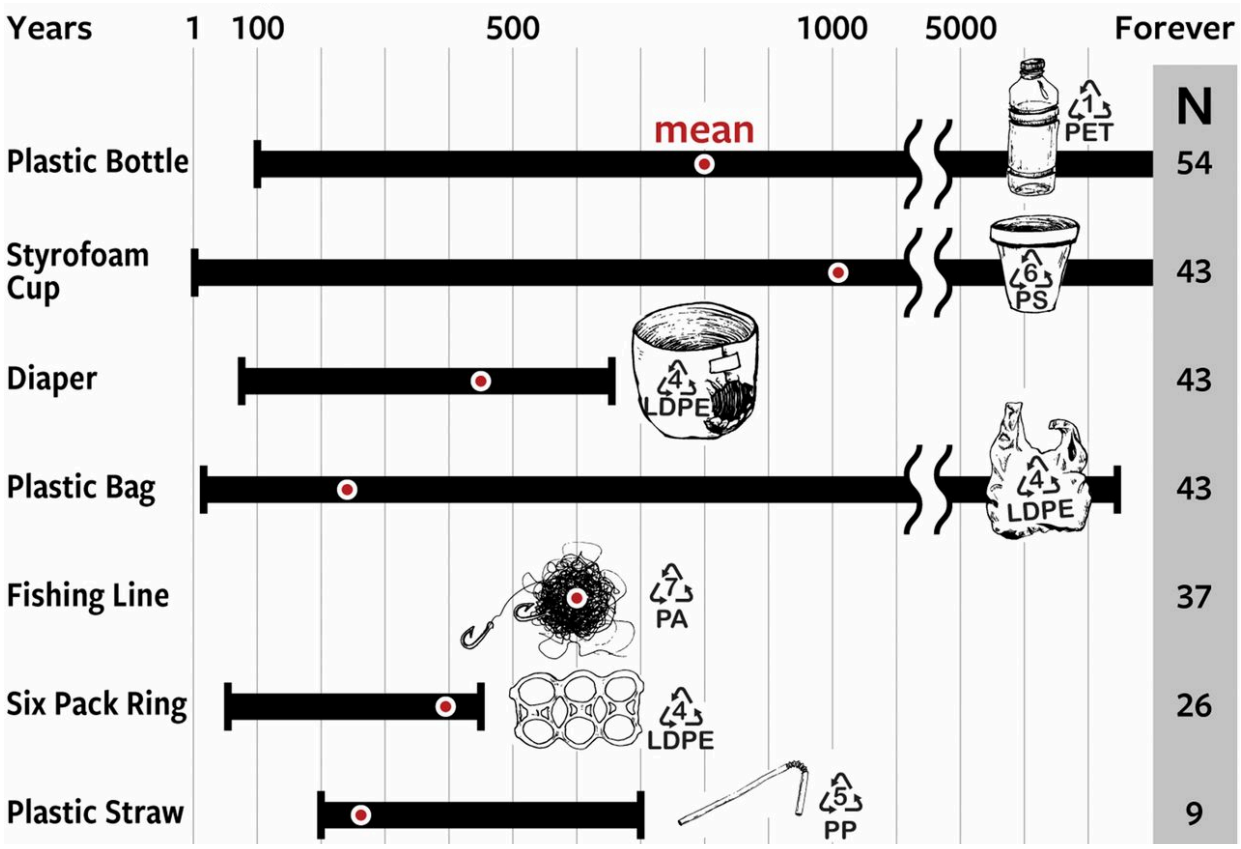


Figure 1.3: Reported lifetimes of common plastic goods. Bars represent the total range of reported lifetimes, and red dots indicate the mean reported lifetime. Column on the right labeled "N" displays the number of data points; sources include governmental agencies, nongovernmental organizations, peer-reviewed journals, college textbooks, and more. Reproduced from Ward et al. (2020) [11].

eating actual food and causing them to gradually starve [13]. Furthermore, plastics are often made with chemical additives (e.g. bisphenol-A, bis(2-ethylhexyl) phthalate, etc.); these chemicals leach out of plastic waste as it degrades, altering and disrupting the surrounding environment [14].

When larger waste breaks down to the microscale or nanoscale, it becomes easily ingested by wildlife. The smaller size of these plastics increases their bioavailability and enables them to interfere with living organisms' health at a cellular level [15]. Microplastics' high surface area to volume ratio makes them excellent carriers for pollutants and toxic additives, inducing sickness when consumed by animals [16]. Microplastics can also make their way into the soil, where they have been observed to change the soil environment and stunt plant growth [17]. For humans, microplastics in our food and air have been demonstrated to induce cytotoxicity, metabolism disruption, DNA damage, myocardial infarction, stroke, and cancer [18], [19]. In 2022, it was estimated that 80% of the human population could have microplastics circulating in their bloodstream [20].

1.2 Plastic Pollution Policies

Given this extensive list of concerns, it is imperative to find an environmentally-friendly solution to the plastic pollution issue. Unfortunately, pollution is a difficult problem to solve. Firstly, pollution is a classic example of a negative externality, wherein the actions of an individual indirectly impose costs on others. This diffusion of personal responsibility encourages actors to make inefficient and irrational choices, prioritizing individual convenience at the expense of societal welfare [21]. Also, the negative impacts of plastic pollution primarily affect beings and places that are far away and out of sight. This makes it difficult to take action, since any party which does so incurs all the costs of the action while the benefits are distributed

globally [22]. Finally, as with many environmental issues, the greatest benefits of plastic pollution reduction lie in the future, but the costs must be paid upfront [23]. All these factors make plastic pollution an especially intractable issue.

Governmental regulation represents one potential method to ameliorate plastic pollution. Broadly speaking, plastic pollution policies fall into three categories: regulatory, economic, and informative. Regulatory instruments are rules of an obligatory nature which allow or disallow certain actions (e.g. directives to capture plastic litter, bans on a specific type of plastic, etc.). Economic instruments utilize the market to incentivize responsible plastic usage (e.g. subsidies to support plastic recycling programs, higher taxes for poor plastic stewardship, fees on single-use plastics, etc.). Finally, informative instruments aim to display and disseminate educational materials in order to change consumer behavior (e.g. research, awareness campaigns, recyclability labeling, etc.) [24].

As plastic usage has intensified, the number of policy initiatives using these instruments to combat plastic pollution has also risen. A growing number of international policies have been aimed at reducing plastic pollution, although most of them are non-binding and structured as plans or recommendations instead of specific commitments [25]. Furthermore, many international policies are aimed at preserving the marine environment and marine wildlife, but do not specifically mention plastic pollution [25]. Accordingly, 28 international policies have been laid out since 2000, but only two general targets have been set: the 2010 Aichi target to reduce pollution broadly, and the UN Sustainable Development Goal 14 target to reduce marine pollution [24]. In 2022, the United Nations Environment Assembly resolved to pass an international, legally binding agreement by 2024. While this agreement is still in the process of drafting and negotiations, it is meant to coordinate and accelerate international action on the

entire life cycle of plastic pollution, from production and product design to proper waste management [26]. If successful, this agreement would represent a great advancement in the effort to combat plastic pollution.

At the national level, regulatory bans (e.g. prohibiting specific pollutants or specific plastic goods) have been by far the most common instruments used to control plastic pollution. According to a survey of 147 national policy responses across the world, national governments were 3.5 times more likely to use regulatory instruments than economic instruments, and 3 times more likely to use regulatory instruments than information instruments **(Figure 1.4)** [24].

Due to their somewhat qualitative nature, it can be difficult to find data on the efficacy of pollution policies. The topic with perhaps the most available data is legislation surrounding plastic bags, including bans and levies. When surveying plastic bag regulation, all types of instruments were shown to induce a short-term reduction in plastic bag usage. However, in the long-term, behavior was more difficult to change. Economic instruments often lost their

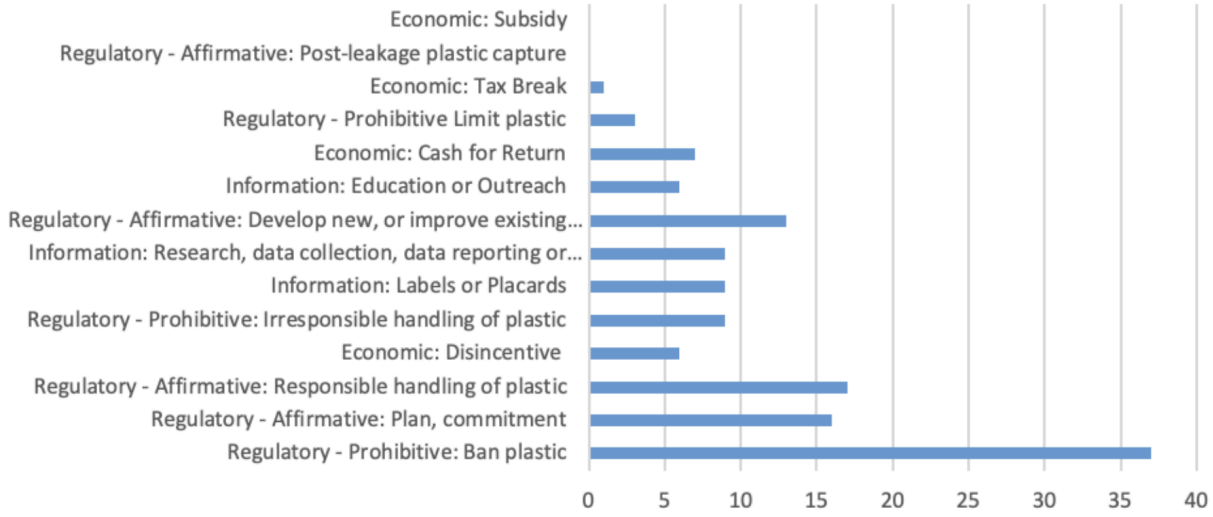


Figure 1.4: Frequency of regulatory instruments used to address plastic pollution. A review of instruments used by national governments, specifically aimed at land-based sources of macroplastic pollution. Policies were taken from Duke University's Plastics Policy Inventory [27]. Reproduced from Karasik et al. (2020) [24].

effectiveness over time due to a phenomenon known as the “rebound effect”, wherein people became accustomed to the fees and slowly increased their consumption back to normal levels [28]. On the other hand, policy instruments should theoretically retain their effectiveness. Yet in several cases, initially-successful policy instruments were subsequently lessened by future policy changes, leading to a rebound in plastic bag consumption [29]. In general, both types of regulation have been demonstrated to be effective in reducing plastic use; bans are likely the more effective tool, but often politically infeasible to pass and/or enforce [30]. Although this data only reflects plastic ban legislation, it reflects an important trend relevant to other plastic pollution issues: specific and binding regulatory instruments may be the most effective tools, but it can be difficult to garner the political momentum necessary to pass and maintain these policies in the long-term. Instead, it may be more helpful to think economically—by developing cost-effective and environmentally friendly materials that can serve as an alternative to traditional plastic.

1.3 Biopolymers as a Potential Solution

While governmental policies represent one pathway to combat the plastic pollution problem, another approach lies in technological innovation. Developing alternative materials that are environmentally friendly while being as functional and cost effective as conventional plastics would allow consumers to transition away from the status quo without any need for legislation. Given that more than a third of plastic consumption is due to packaging applications, finding a substitute for plastic packaging could drastically reduce plastic’s environmental impact [31]. These alternative plastics should have the same material properties as current plastics in terms of mechanical strength, plasticity, water resistance, and more. Furthermore, to address the plastic pollution problem, alternative plastics should be biodegradable, so as to minimize their long-term

impact on the environment. Finally, ideal alternative plastics should be similar or even lower in cost when compared to conventional plastics; this cost parity provides a market-based incentive to abate plastic pollution, reducing the burden on governments.

Biopolymers present one possible alternative to conventional plastics. The vast majority of conventional plastics are made from petroleum-based polymers in a process that burns fossil fuels and releases greenhouse gas emissions. In order to produce plastics, raw materials (e.g. crude oil, natural gas, and coal) are extracted, vaporized using heat and segregated into its components by boiling point and weight. The resulting refined material then undergoes a process called “cracking”, in which high temperature, pressure, and sometimes chemical catalysts are used to convert refined petroleum products into their monomeric forms. In the final step, the monomers are linked together to form polymers, often using catalysts to initiate the reaction [32]. All of these steps add up to form an energy-intensive, environmentally-destructive manufacturing process.

Recently, however, alternative plastics made from bio-based polymers have begun to garner attention for their environmental benefits. Broadly speaking, bio-based polymers can be broken down into three major classes: A) those made directly from biomass (e.g. starch, cellulose, chitosan, and alginate), B) those bio-synthesized using microorganisms and plants (e.g. polylactic acid and polyhydroxyalkanoates), and C) conventionally oil-based polymers prepared from alternative bio-sourced monomers (e.g. polyethylene and polyethylene terephthalate) [33]. Bio-based polymers have several advantages when compared to their petroleum-based counterparts. Firstly, they are generated from renewable sources, whereas petroleum-based plastics rely on nonrenewable sources which are quickly being depleted. Moreover, these renewable sources have carbon-fixing properties, which serve to decrease the total greenhouse

gas emissions produced by plastic manufacturing. Finally, many class A and class B bio-based plastics are biodegradable; when discarded, they fully decompose in a matter of months as opposed to centuries. Class A bio-based polymers are particularly appealing because they are less expensive and more widely available than class B bio-based polymers, which require more complex manufacturing processes [34]. Class A bio-based polymers are also generally even more readily degradable than class B bio-based polymers; for instance, the popular class B polymer polylactic acid (PLA) is actually nondegradable in soil conditions at ambient temperature, and requires special conditions like UV radiation or PLA-degrading enzymes to degrade [35]. Therefore, a class A bio-based polymer material could potentially serve a cost-effective and environmentally friendly alternative to the petroleum-based plastics commonly used today.

Although class A biopolymers have many benefits, they nevertheless also suffer from several key drawbacks. Firstly, class A biopolymers typically have weak mechanical properties which limit their functionality. Films made from starches, for instance, display tensile strengths in the 1-20 MPa range [36]. In comparison, films made from the most common plastic packaging materials like low-density and high-density polyethylene have tensile strengths in the 20-59 MPa and 14-200 MPa range, respectively [37], [38]. On their own, class A biopolymer films also tend to be brittle and fragile, prone to cracking and breaking [36]. Therefore much current research focuses on improving mechanical properties to make them similar to conventional plastics, usually by incorporating additives, blending with other polymers, and/or chemical crosslinking. In these cases, it is important to note that such modifications can often introduce toxic substances or inhibit the biodegradability of the resulting materials [39]. Class A biopolymers also suffer from inherent batch-to-batch variation. Since they are derived directly from biomass, differences

in the origin, environmental conditions, and processing of the starting raw material can cause irregularities in their properties, making class A biopolymer products less consistent than their synthetic counterparts [40]. Lastly, while class A biopolymers may be less expensive than other types of bio-based polymers, they are still several times more expensive to manufacture than conventional plastics [41]. This cost differential is expected to decrease as the biopolymer industry becomes larger and more developed, but it is important to keep in mind, as price parity will be crucial for encouraging consumers to adopt biopolymer plastic alternatives.

1.4 Shrilk

Shrilk is a biomimetic material modeled after the structure of insect, crustacean, and mollusk exoskeletons [42]. In these organisms, the bulk of the exoskeleton's thickness is taken up by the procuticle, a composite material containing chitin nanofibers embedded in a proteinaceous matrix [43]. Research indicates that cuticular chitin and protein fibers organize into a complex structure: the chitin forms long, antiparallel chains; the chains are wrapped with proteins to form nanofibrils; clusters of nanofibrils join to form chitin-protein fibers; aligned fibers organize into horizontal planes; and horizontal planes are stacked in a twisted plywood structure **(Figure 1.5)** [43]. These stacks of horizontal planes are evident in SEM imaging of the cuticle as distinct chitin-protein layers **(Figure 1.5)** [44].

Shrilk mirrors this natural biological arrangement by stacking two distinct layers of class A biopolymers, chitosan and silk fibroin, on top of each other in order to create a laminate with phase-separated polysaccharide and protein layers [42]. The name "Shrilk" is a portmanteau of the two words, "shrimp" (because chitosan is often isolated from shrimp shells) and "silk" (from silk fibroin). According to the original publication, "Unexpected Strength and Toughness in Chitosan-Fibroin Laminates Inspired by Insect Cuticle," this laminate displayed an ultimate

tensile strength of 119 MPa—two times that of pure chitosan, its stronger component, and over ten times that of a chitosan-silk fibroin blend—without the need for any covalent crosslinking or other intense chemical modifications. It also demonstrated a toughness of ~2.6 J/cm^3, roughly 1.5 times the toughness of chitosan alone. Furthermore, Shrilk was optically clear; this property set it apart from current plastic substitutes like paper, which are not transparent. Accordingly, Shrilk was presented as a promising biodegradable alternative to traditional plastic packaging materials

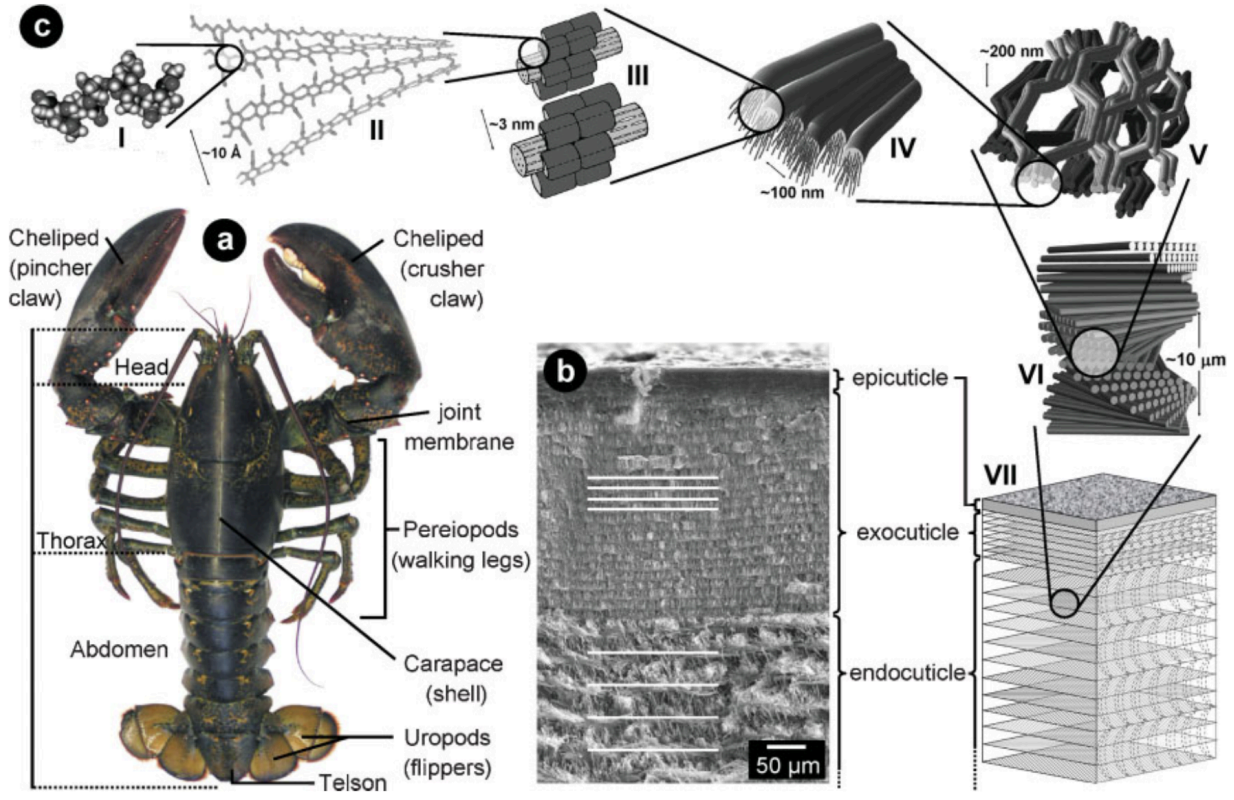


Figure 1.5: Microstructure of lobster cuticle. (a) Diagram of the American lobster *Homarus americanus*. **(b)** SEM image of the lobster cuticle cross-section, illustrating the laminar arrangement of chitin-protein fibers. **(c)** Diagram of the different hierarchies of organization: (I) N-acetyl-glucosamine molecules; (II) anti-parallel chains of α-chitin; (III) 18 to 25 chitin chains wrapped with proteins, forming nanofibrils; (IV) clusters of nanofibrils, forming chitin-protein fibers; (V) horizontal planes of fibers with fibers oriented in the same direction; (VI) horizontal planes stacked in a twisted plywood structure; (VI) the three-layered cuticle. Reproduced from Muthukrishnan et al. (2020) [45].

[42]. Given that an estimated 40% of plastic waste comes from single-use packaging, the potential benefit of developing a more sustainable packaging material is immense [46].

However, following its publication, Shrilk has suffered from reproducibility issues, making it difficult to market as a viable plastic alternative. Attempts to fabricate Shrilk have been unable to reliably recapture the same tensile strength and toughness reported in the initial article. Thus, a need exists to investigate whether chitosan-silk fibroin laminates truly result in unexpected strength and toughness and can therefore serve as a feasible biodegradable alternative to plastic packaging. Also, while Shrilk reports high tensile strength and toughness, these properties are insufficient to make it comparable to conventional plastics. For instance, Shrilk has low elasticity and loses its tensile strength when wet, so effort should also be focused on optimizing Shrilk's material properties to match industry standard benchmarks. Finally, the original method for fabricating Shrilk is rather time and cost intensive, largely due to the processing required to create aqueous silk fibroin. Therefore, this thesis will also investigate potential proteins that could replace silk fibroin in Shrilk, making it easier and less expensive to produce.

1.5 Specific Aims

Aim 1: Fabricate and characterize structure-function properties of Shrilk

While improved mechanical properties in chitosan-silk fibroin laminates were first reported in 2012, many attempts have been made to replicate these findings with limited success. However, we hypothesize that the laminate structure does indeed result in higher tensile strength and toughness, and that previous failures to replicate were due to methodological discrepancies. Therefore the first goal of this thesis is to recreate a chitosan-silk fibroin laminate which demonstrates superior mechanical performance to chitosan, silk fibroin, and chitosan-silk blends.

Successful Shrilk films should also be optically clear. Scanning electron microscopy, FTIR spectroscopy, and Raman spectroscopy will be used to better understand the structure and mechanism behind Shrilk's mechanical properties. A degradation study will be conducted to quantify Shrilk's biodegradation timeline.

Aim 2: Investigate whether silk fibroin can be substituted with alternative proteins

Silk fibroin has many unique advantages, but it is also relatively expensive and difficult to process. Making aqueous silk fibroin from raw silk takes four days and typically requires harsh chemicals like lithium bromide (LiBr), generating large amounts of hazardous liquid waste. We hypothesize that the increased tensile properties resulting from laminate films could be reproduced using a protein that is similar to silk fibroin. Thus the next goal of this thesis is to investigate whether alternative protein sources—namely, collagen, keratin, and casein—can impart the same mechanical strength as silk fibroin when combined with chitosan in laminate form. If successful, switching to a substitute protein could drastically simplify Shrilk's manufacturing process, cutting down on both time and waste production.

Aim 3: Increase Shrilk's tensile properties and water resistance capabilities

In order to be a successful alternative to plastic, Shrilk needs to hit certain material benchmarks. Shrilk should have tensile strength, toughness, and elongation at break comparable to commercial plastics. Furthermore, given the diversity and versatility found in commercial plastics, Shrilk's properties should be tunable so that it can serve a variety of different applications. We hypothesize that increasing the molecular weight of chitosan, decreasing the thickness of Shrilk, and increasing the number of layers could heighten Shrilk's tensile strength and toughness. We also hypothesize that adding glycerol to the chitosan and silk could increase Shrilk's flexibility and elongation at break. Finally, although Shrilk has good mechanical

properties when dry, it is much weaker when wet. This is an important drawback since water resistance is vital for packaging materials, which routinely come into contact with water and water vapor. We hypothesize that covering Shrilk in a hydrophobic coating could prevent Shrilk from losing its tensile strength when wet. In summary, the goal of this section is to make alterations to Shrilk and optimize its properties so that it can function similarly to conventional plastic packaging materials, even after coming into contact with water.

Final Outcome:

Successful Shrilk films should have tunable tensile properties in ranges comparable to common plastic packaging materials. Films should be optically clear, water resistant, and readily biodegradable. If possible, silk fibroin should be substituted with an alternative protein that is less expensive, less energy intensive, and easier to process. A potential mechanism behind Shrilk's mechanical properties should be proposed and backed up by analytical chemistry techniques. **Ultimately, the goal of this thesis is to provide a comprehensive overview of the structure and properties of Shrilk and other chitosan-protein laminates in order to determine whether they can serve as feasible alternatives to conventional plastic packaging.**

2. Shrilk Fabrication and Characterization

2.1 Background on Chitosan

Chitosan, one of the two components of Shrilk, is a derivative of chitin, the second most abundant biopolymer on Earth. Chitin is a structural amino-polysaccharide polymer composed of N-acetylglucosamine units connected by β-(1-4) glycosidic bonds **(Figure 2.1)**. This structure gives chitin exhibit unique physical, chemical, mechanical, and optical properties [47]. It is commonly found in crustacean and insect exoskeletons and the cell walls of fungi, where it confers mechanical strength. Conveniently, crab and shrimp shells, which would otherwise become marine waste, provide an abundant source of chitin for industrial processing. Additionally, as a class A biopolymer, chitin is renewable and biodegradable. As a result, chitin has garnered much interest in recent scientific literature. However, strong hydrogen bonds between the acetyl, amino and hydroxyl groups in chitin make it insoluble in common solvents (e.g. water, mild acidic or basic solutions, organic solvents, etc.) [48]. As a result, its

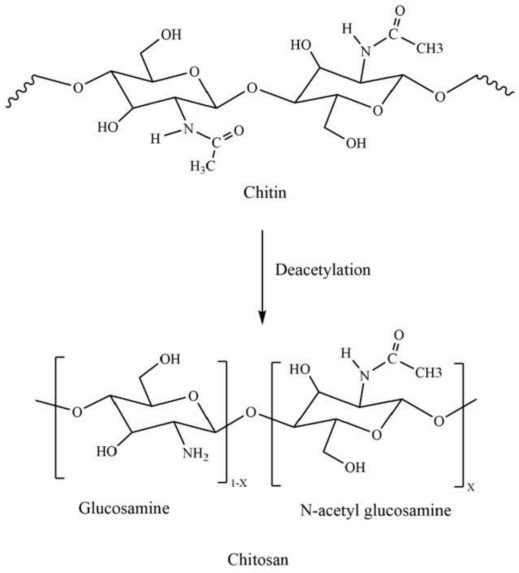


Figure 2.1: Structure of Chitin and Chitosan. Following deacetylation, a percentage of N-acetylglucosamine units are converted into simple glucosamine units. Reproduced from Chandrasekaran et al. (2020) [49].

deacetylated form, chitosan, is more commonly used **(Figure 2.1)**. Chitin can be deacetylated to varying degrees; typically, greater than 50% deacetylation is considered chitosan and less than 50% deacetylation is still considered chitin. Chitosan dissolves readily in mildly acidic solvents such as 0.1M acetic acid, through the protonation of the $–NH_2$ group on the C-2 position [50].

This increased solubility allows chitosan to take on a wide range of applications. For example, chitosan has received much attention in the biomedical space due to its biocompatibility, degradability, and antibacterial properties. It can be used as a flexible drug delivery system or as scaffolding for tissue engineering to promote cell attachment, proliferation, and differentiation [51], [52]. Chitosan-based materials take on many forms, including films, hydrogels, porous structures, and fibers, which can be loaded with drugs, peptides, cells, and more [53]. Since chitosan is a positively charged biopolymer, it interacts with negatively charged bacterial cell walls, disrupting their structure and inducing bacterial death [54]. Importantly, chitosan can readily be blended with nanoparticles or other polymers to tune its mechanical, chemical, and degradation properties to suit particular use cases [55].

Many of these qualities—biodegradability, nontoxicity, optical clarity, and antimicrobial activity—also make chitosan films uniquely suited to be used as a packaging material [56]. Furthermore, chitosan films have superior mechanical properties when compared to many other class A biopolymers. Reported tensile strengths of pure chitosan films range from roughly 10-80 MPa [57], [58]. This variation may be attributable to different chitosan sources, deacetylation percentages, molecular weights, and tensile testing procedures. By contrast, most films made from other proteins including zein, soy protein, and gelatin, have tensile strengths less than 10 MPa [59]. When tested as a form of food packaging, chitosan films have been shown to increase

the shelf-life of fruits, vegetables, and meats by weeks, reduce microbial growth, and increase antioxidant activity [60].

Scientific interest in chitosan is reflected in the growth of industrial chitosan production. In 2022, Grand View Research valued the global chitosan market size at $10.88 billion, and projected that the chitosan market would expand at a compound annual growth rate (CAGR) of 20.1% from 2023 to 2030 [61]. This immense growth rate is attributed to the high demand for chitosan due to its biomedical and environmental applications.

2.2 Background on Silk Fibroin

Silk, the second component of Shrilk, has been used in the textile industry for over 5000 years. Although many species of insects produce silk, including spiders, bees, and moths, the most common source of silk is the *Bombyx mori* silkworm [62]. *B. mori* moths start producing silk at around day 26 of their life cycle, roughly halfway through their lifespan. They use this silk to spin a cocoon, in which they metamorphose for about two weeks before emerging as an adult moth [63]. *B. mori* silk fibers can thus be harvested at the cocoon stage, before adult moths emerge.

Silkworm cocoons are composed of two parts: silk fibroin and sericin. Silk fibroin is a fibrous protein composed of a ~350 kDa heavy chain polypeptide and a 25 kDa light chain polypeptide, linked by a disulfide bond, as well as another 25 kDa polypeptide which interacts hydrophobically with the heavy and light chains [64]. Sericin is an adhesive protein that binds silk fibroin strands together and maintains the shape of silkworm cocoons [62]. Silk fibroin can be isolated from silkworm cocoons through the degumming process; boiling cocoons in an alkaline solution dissolves the sericin and yields purified silk fibroin [65]. Crystalline regions consisting of Gly-X repeats make up 94% of silk fibroin's heavy chain, where X is Ala (65%),

Ser (23%), and Tyr (9%) **(Figure 2.2)** [66]. *B. mori* silk fibroin's unique composition allows it to switch between two phases: Silk I, wherein the polypeptide chain is dominated by α-helices and random coils, and Silk II, wherein the polypeptide chain is organized into antiparallel β-sheets connected by strong hydrogen bonds and van der Waals forces [67]. The transition from Silk I to Silk II can be induced using many methods, including methanol treatment, heat, and shear stress [65].

As a result of its structural organization, silk has engendered much scientific interest. Like chitosan, silk fibroin is biodegradable, nontoxic, and nonimmunogenic [68]. It has high tensile strength and good oxygen barrier properties [69]. Furthermore, its mechanical and chemical characteristics can be easily tuned by manipulating factors like protein concentration,

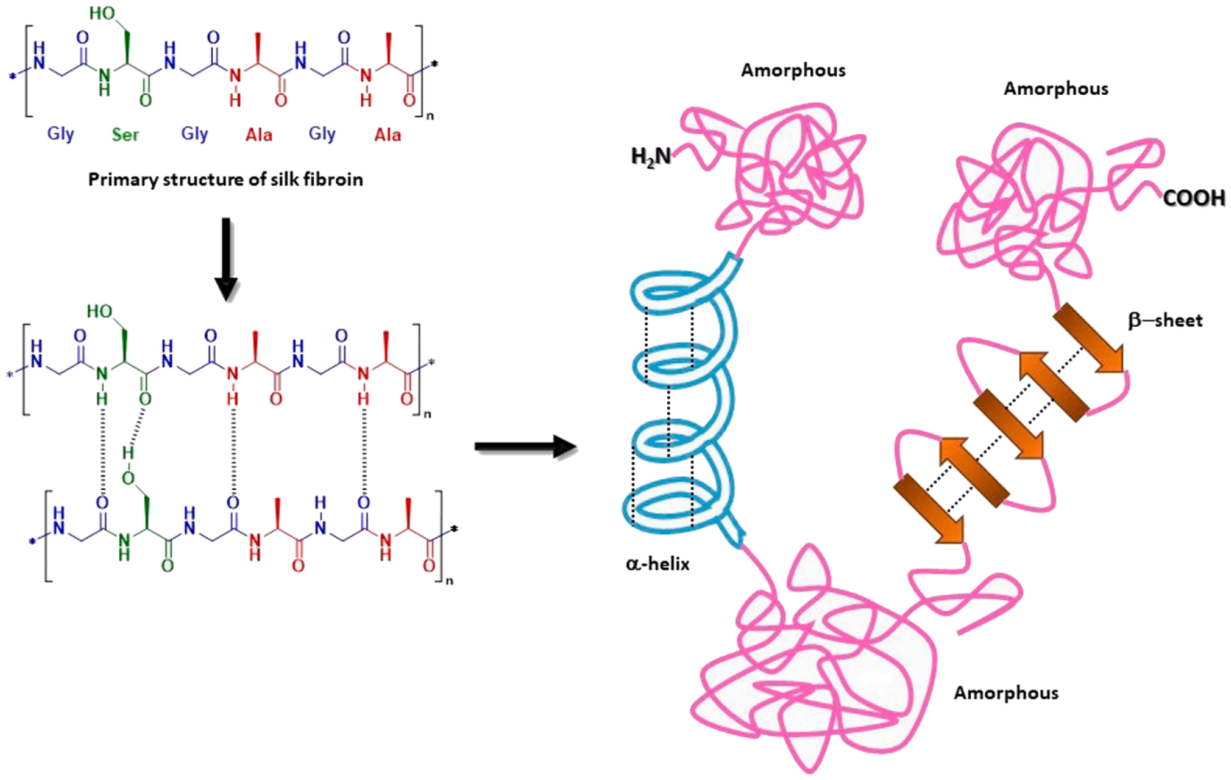


Figure 2.2: Structure of Silk Fibroin. The primary structure of silk fibroin consists of many Gly-X repeats. Through hydrogen bonding, silk fibroin self-organizes into α-helices, β-sheets, and amorphous random coils. Reproduced from Elhadad et al. (2022) [70].

pH, temperature, and mechanical stress to induce specific conformational changes between Silk I and Silk II [71]. In the biomedical space, silk fibroin has been formed into gels, films, sponges, and fibers to create tissue scaffolds, drug delivery agents, sutures, and more [68]. Silk fibroin has also been investigated as part of optical devices and electronic devices, and even as an adsorbent for water pollution [72]. Its use in packaging is somewhat less studied than chitosan, perhaps due to its low flexibility, water solubility, and susceptibility to microbial contamination [69]. However, these weaknesses can be circumvented through various modifications, such as introducing plasticizers and crosslinkers.

2.3 Film Characterization Techniques

In this section, chitosan-silk fibroin laminates were fabricated according to the initial method laid out by Fernandez and Ingber in 2012 [42]. After making these films, a number of methods were used to analyze their characteristics. Scanning electron microscopy (SEM) was used to visualize Shrilk films. Tensile testing was performed to measure Shrilk's mechanical properties—in particular, tensile strength, toughness, and elongation at break, as these are essential characteristics for plastic packaging materials. Fourier Transform Infrared (FTIR) spectroscopy and Raman spectroscopy were used in order to help elucidate the mechanism behind the laminate's increased strength and toughness: Fourier Transform Infrared Spectroscopy (FTIR) to analyze the functional groups at the surface of the films, and Raman Spectroscopy to track the functional groups as they changed across the chitosan-silk fibroin interface. Lastly, a biodegradation study was performed to quantify Shrilk's inherent degradation timeline. Taken together, these methods aimed to present a comprehensive summary of Shrilk's properties, as well as propose a potential mechanism to explain the source of Shrilk's strength and toughness.

2.4 Methods

Chitosan Films

Chitosan (Sigma Aldrich, USA) was dissolved at 1% w/v in 1% v/v acetic acid and stirred until homogenous. 6.75 mL of chitosan solution was poured into 6 cm x 6 cm molds, with glass bottoms and polyethylene walls, and allowed to evaporate overnight at 50°C. Chitosan films were then submerged in 1M NaOH for 10 minutes to neutralize protonated amino groups and washed in Milli-Q water. Films were dried at room temperature and removed from the glass molds using tweezers and an X-Acto knife.

Silk Fibroin Films

B. mori silk cocoons (Treenway Silks, USA) were boiled in 0.02M Na_2SO_3 for 30 minutes for degumming, then washed with Milli-Q water and dried at room temperature overnight. To prepare an aqueous solution, 20% w/v silk fibroin was dissolved in 9.3M LiBr (Sigma Aldrich, USA) and incubated for four hours at 60°C, until no distinct silk fibers were visible. The resulting solution was dialyzed against Milli-Q water using 12-14 kDa dialysis tubing to remove LiBr, with five water changes over 48 hours [73]. Dialyzed silk fibroin solution was centrifuged to remove impurities. To calculate solids percentage, 5 mL of silk fibroin solution was poured into a pre-weighed glass Petri dish and dried overnight at 50°C. The difference in weight, divided by 5, was equal to the amount of silk fibroin protein per mL.

To cast silk fibroin films, the amount of silk fibroin solution corresponding to 0.135 grams of fibroin protein was poured into the same 6 cm x 6 cm molds and dried overnight at 50°C. Resulting films were submerged in methanol for 30 minutes to encourage beta sheet formation, then washed with Milli-Q water. Films were dried at room temperature.

Shrilk Films

First, chitosan films were formed as described above. Subsequently, a layer of silk fibroin was cast on top of the chitosan, and then treated with methanol as described above. Shrilk films were dried at room temperature.

Scanning Electron Microscopy

Shrilk films were prepared as previously described, then manually torn into ~1 cm x 1 cm sheets. The sheets were mounted to aluminum pin mounts with conductive carbon tape, sputter-coated with gold, and imaged with a Tescan Vega GMU scanning electron microscope (Tescan, Czech Republic).

FTIR Spectroscopy

Chitosan, silk fibroin, and Shrilk films were prepared as previously described and analyzed using the Nicolet iS50 FTIR Spectrometer on the mid-IR ATR setting (Thermo Fisher, USA). The spectra were processed using an advanced ATR correction and a baseline correction.

Raman Spectroscopy

Chitosan, silk fibroin, and Shrilk films were prepared as previously described and analyzed using the XploRA Plus Raman Spectrometer (Horiba, Japan). In order to avoid collecting signals from any extraneous material, rectangles were cut out of two pieces of cardstock, and the pieces of cardstock were stacked on top of each other to form a small viewing window. The film was then mounted in between the two layers. Acquisition time was adjusted to attain a signal-to-noise ratio of 10:1, and two accumulations were performed for each scan. For the Shrilk film, a map was collected along the z-axis, beginning and ending 5 μm above and below the film boundary. Baseline correction and other data processing was performed using Lab Spec 6.

Stress-Strain Measurements

Chitosan, silk fibroin, and Shrilk films were cut into 1.5 cm x 5 cm rectangles for tensile testing. Film thickness was calculated as the average of three thickness measurements, taken using digital calipers. Ramp to failure testing (0.1 mm/s) was carried out using a 100 N load cell on an Instron 68TM-10 instrument (Instron, USA). Tensile strength was determined by the maximum force divided by the cross-sectional area; modulus of toughness was determined by the integral of the stress-strain curve until failure; elongation at break was determined by the displacement divided by the gauge length.

Soil Burial Method

In order to simulate a landfill condition in the laboratory, a cylindrical glass container was filled with compost (Espoma, USA) to a height of 5 cm. 2 cm x 2 cm Shrilk films were placed on the soil, and covered with 5 cm of more soil. The glass container was lightly watered every day to maintain soil moisture. Films were taken out of the glass cylinder after two and four weeks, gently cleaned of debris, dried in the oven, and weighed to measure mass loss.

Data Analysis and Presentation

MATLAB was used to analyze stress-strain data and create graphs. Error bars were used to indicate the standard deviation of collected data. One-way ANOVA tests were carried out using the XLMiner Analysis ToolPak extension in Google Sheets. Two-tailed Student's T-tests and F-tests were carried out using native Google Sheets functions. Biorender was used to create process diagrams. Figures were compiled using Microsoft Powerpoint.

2.5 Results

Shrilk films were fabricated according to the protocol laid out by Fernandez and Ingber (**Figure 2.3a)** [42]. SEM imaging confirmed that Shrilk films had two distinct layers, chitosan

and silk fibroin **(Figure 2.3b)**. The films were ~50 μm thick, with chitosan and silk fibroin each taking up approximately half the thickness. Shrilk films were optically clear to the extent that words could be distinguished across the film, whereas chitosan-silk fibroin blends were opaque and unable to be seen through **(Figure 2.3c-d)**. Shrilk films were readily biodegradable; when buried in compost, they lost 54% of their mass after 2 weeks and 84% of their mass after 4 weeks **(Figure 2.3e)**. When chitosan controls, silk fibroin controls, Shrilk films, and chitosan-silk

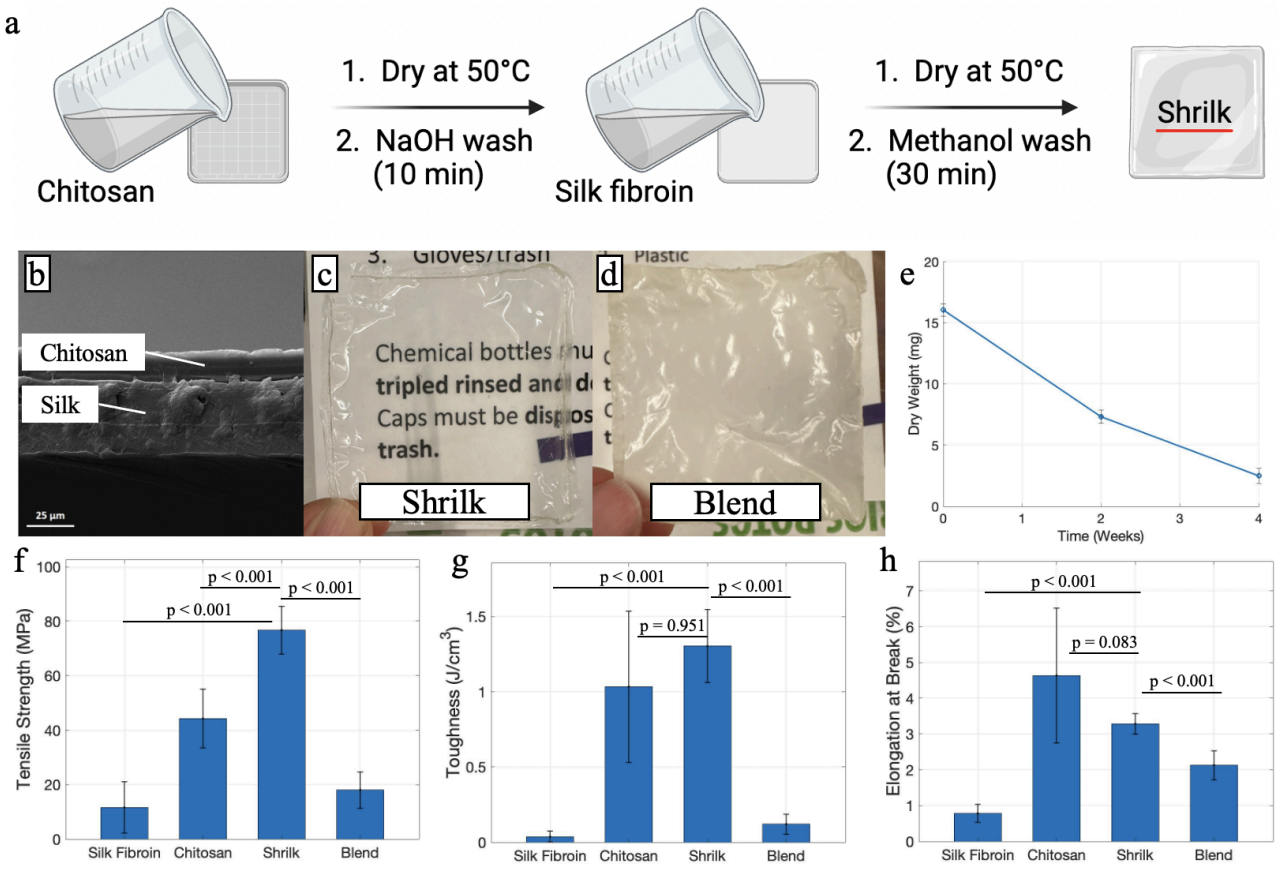


Figure 2.3: Shrilk. Shrilk films were fabricated according to the protocol illustrated in **(a)**. **(b)** SEM imaging confirmed that Shrilk films consisted of two distinct layers, chitosan and silk fibroin. **(c)** Shrilk films were optically clear, unlike **(d)** chitosan-silk fibroin blends, which were opaque. **(e)** Shrilk films degraded by more than 50% after 2 weeks, and nearly 90% after 4 weeks. Silk fibroin controls, chitosan controls, Shrilk films, and chitosan-silk fibroin blends were compared for **(f)** tensile strength, **(g)** toughness, and **(h)** elongation at break. Mean values are shown and error bars represent ± standard deviations (n = 6-8 samples per group). Pairwise p-values were calculated using two-tailed Student's T-tests.

fibroin blends were compared using tensile testing, one-way ANOVA revealed a significant difference between experimental groups ($p < 0.001$). Specifically, Shrilk had the highest mean tensile strength of 76.7 MPa: 73% greater than the chitosan control's tensile strength of 44.3 MPa ($p < 0.001$), 561% greater than the silk control's tensile strength of 11.6 MPa ($p < 0.001$), and 326% greater than the chitosan-silk fibroin blend's tensile strength of 18.0 MPa ($p < 0.001$) **(Figure 2.3f)**. In terms of toughness, Shrilk films were significantly tougher than silk and blended films ($p < 0.001$), but there was no statistically significant difference when compared to chitosan controls ($p = 0.951$) **(Figure 2.3g)**. However, Shrilk films had less variable toughness than chitosan controls ($p = 0.002$) **(Figure 2.3g)**. Finally, silk fibroin control films had lower elongation at break values than all three other groups ($p < 0.001$) **(Figure 2.3h)**. Chitosan control films had the highest mean elongation at break value, but there was no statistically significant difference between Shrilk films and chitosan control films ($p = 0.083$) **(Figure 2.3h)**.

In order to better understand the mechanism behind Shrilk's increased tensile strength, FTIR spectroscopy and Raman spectroscopy were performed. The FTIR spectra of a chitosan control film and the chitosan side of a Shrilk film looked very similar when superimposed, although the Shrilk chitosan had higher peaks in the 1300-1700 cm^{-1} and 2800-3500 cm^{-1} region **(Figure 2.4a)**. The 1300-1700 cm^{-1} region contains peaks related to C=O, NH_2, and NH-bending vibration in chitosan's amide and amino groups, and the 2800-3500 cm^{-1} region contains peaks related to the OH hydroxyl group and CH_2 stretching in the pyranose ring [74]. The FTIR spectra of a silk fibroin control film and the silk side of a Shrilk film also looked very similar when superimposed **(Figure 2.4b)**. The only noticeable difference was slightly lower peaks at 1530 cm^{-1} and 1630 cm^{-1} from the Shrilk film, both of which correspond to the presence of β-sheets **(Figure 2.4b)** [75]. Since FTIR spectroscopy only penetrates the first ~0.5 μm of a substance,

Raman spectroscopy was used to track the presence of functional groups as they changed throughout the thickness of the film. Raman spectroscopy of chitosan and silk control films was performed to generate a peak table containing each material's characteristic peaks **(Figure 2.5a-b)**. Then, the intensities of peaks unique to each material—the 1374 cm^{-1} for chitosan and the amide I peak at 1665 cm^{-1} for silk fibroin—were plotted as a function of depth on the z-axis

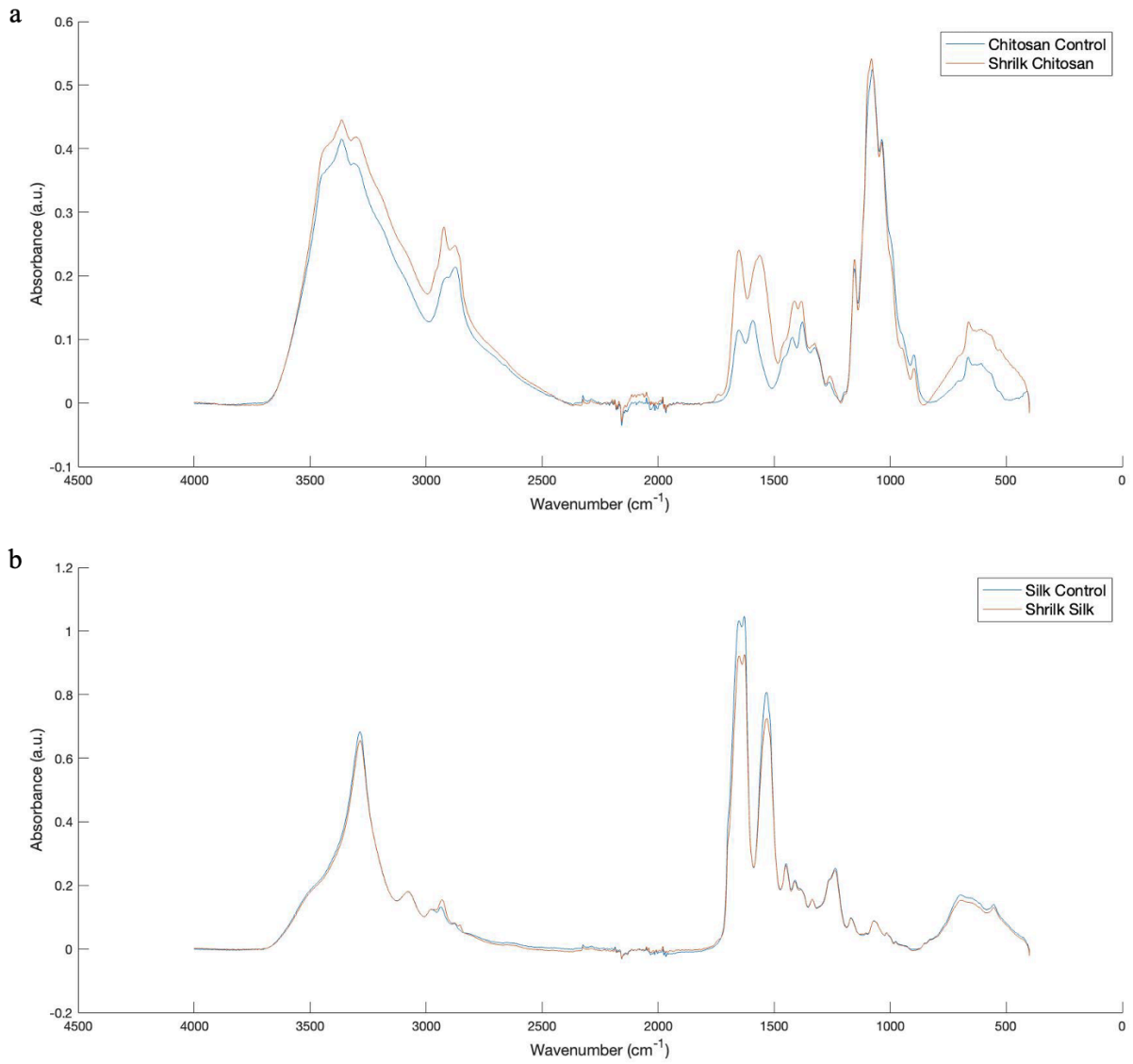


Figure 2.4: FTIR spectra of Shrilk and control films. (a) Overlaid FTIR spectra of a chitosan control film and the chitosan side of a Shrilk film. **(b)** Overlaid FTIR spectra of a silk fibroin control film and the silk fibroin side of a Shrilk film.

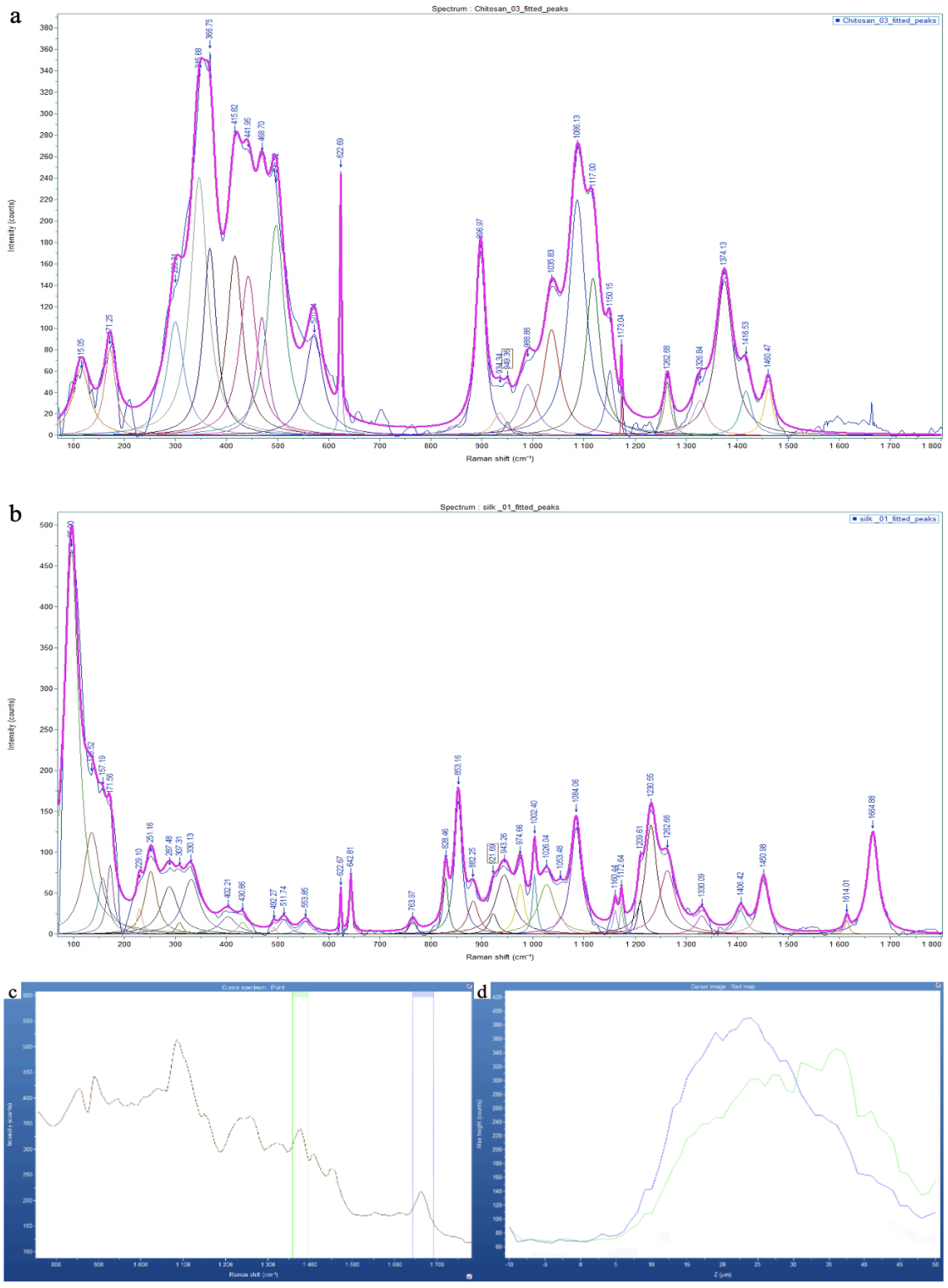


Figure 2.5: Raman spectra of Shrilk and control films. Peak-fitted Raman spectra of **(a)** a chitosan control film and **(b)** a silk fibroin control film. Additionally, the Raman microscope was used to generate a map across the z-axis, in order to track the evolution of certain functional groups highlighted in **(c)** and plot their relative frequencies in **(d)**.

(Figure 2.5c-d). The 1665 cm^{-1} shift from the silk fibroin is known to correspond to the amide I band, but the 1374 cm^{-1} seems to lack an assignment in the literature [76], [77]. The resulting plot showed a significant region of overlap taking up most of the film's thickness, which contained functional groups from both chitosan and silk fibroin. Nevertheless, the two curves peaked roughly 15 μm apart, indicating that one half of the Shrilk film remained dominated by chitosan and the other half by silk fibroin **(Figure 2.5d)**.

2.6 Discussion

This section successfully demonstrated that chitosan-silk fibroin laminates had significantly higher tensile strengths than either chitosan and silk alone, as well as a chitosan-silk fibroin blend. However, contrary to the initial publication, the Shrilk films fabricated here did not display an increased toughness when compared to chitosan control films, although they did demonstrate lower variability. Broadly speaking, tensile strength measures the highest stress that a film can withstand before breaking, whereas toughness measures the amount of energy that a film can absorb before breaking. Since toughness can be calculated as the area under the stress-strain curve, toughness is often correlated with tensile strength; however, they can also be quite unrelated, especially for materials that do not deform very much before breaking [78]. This seems to be the case for the Shrilk films produced in this study: they had relatively low elongation at break values, were unable to deform and dissipate energy, and thus demonstrated low toughness. Unfortunately, the original publication did not contain elongation at break values to serve as a point of reference.

In general, the tensile properties observed here were substantially lower than the numbers reported in the initial Shrilk paper. The original Shrilk films claimed to have a mean tensile strength of 119 MPa and toughness of ~2.6 J/cm^3; in comparison, these Shrilk films had a mean

tensile strength of 77 MPa and toughness of 1.3 J/cm^3 [42]. Chitosan films in the original paper had a tensile strength of ~60 MPa and toughness of 1.8 J/cm^3; in comparison, these chitosan films had a tensile strength of 44 MPa and toughness of 1.0 J/cm^3 [42]. Thus, measured tensile properties were lower across the board. The source of these differences is unclear. One potential explanation is the use of different strain rates when conducting tensile testing. Higher strain rates tend to result in higher measured tensile strengths, and the original paper did not specify a strain rate in the tensile testing methodology [79]. Another potential explanation may be the use of 1% w/v chitosan solution instead of 2% w/v chitosan solution, as the original paper suggested. Throughout the course of experimentation, 1% w/v chitosan was found to be less viscous and easier to work with. Research has shown that the concentration of chitosan solution prior to drying can have a substantial impact on the mechanical properties of the film, as films made from lower percentage chitosan solutions have higher acetic acid content; the acetic acid acts as a plasticizer and creates a looser network structure, leading to lower tensile properties [80]. However, the same paper showed that using a neutralization treatment like NaOH effectively eliminates any difference in tensile properties that may result from variations in chitosan percentage [80]. Therefore this divergence should not have had a large effect on the films' mechanical properties.

Nonetheless, despite the generally lower tensile properties, Shrilk films still proved to be nearly twice as strong as the chitosan films, indicating that the laminar structure indeed has an effect on the material's tensile properties. Shrilk films were also much stronger and tougher than blended films, suggesting that the organized laminar structure increases tensile properties, while the blended structure disrupts polymeric organization and decreases tensile properties. Importantly, the tensile properties demonstrated by these films were still comparable to some

common plastic packaging materials. For instance, one commercial 50 μm-thick polystyrene film reports a tensile strength of ~80 MPa and elongation at break of 3-5%, whereas Shrilk films had a tensile strength of 77 MPa and elongation at break of 3.28% [81]. This similarity demonstrates that Shrilk can serve as a viable alternative to plastic packaging materials. On the other hand, a commercial polyester film can have a tensile strength of ~200 MPa and elongation at break of 140%, and a commercial low-density polyethylene film can have a tensile strength of ~10 MPa and elongation at break of 500% [82], [83]. Therefore, finding ways to increase Shrilk's tensile strength, toughness, and/or elongation at break would help to make it an even more appealing plastic substitute.

Spectroscopy was used to determine the mechanism behind Shrilk's increased tensile strength. FTIR results were not particularly illuminating, as the FTIR spectra of the Shrilk samples and the control films were very similar. This suggests that the Shrilk fabrication process did not significantly change the chemical structure at the film's surface. However, Raman spectroscopy indicated that there was a large region of overlap throughout the film's thickness, where functional groups from both chitosan and silk fibroin were present. Raman spectroscopy results thus suggest that casting silk fibroin on top of chitosan resulted in some mechanical entanglement between the two different biopolymers, which may explain the increased tensile strength. At the same time, the film's optical clarity implies that Shrilk films displayed some crystallinity or structural organization that the chitosan-silk fibroin blends were unable to achieve. It is possible that the relatively drier chitosan film acted as a template for the drying silk fibroin solution as entanglement occurred, encouraging the formation of hydrogen bonds and β-sheets in a more well-ordered manner than would have been possible otherwise.

Finally, Shrilk films demonstrated high biodegradability, losing nearly their entire mass after being buried in compost for four weeks. This finding is encouraging, though not necessarily surprising, as both chitosan and silk fibroin are known to be biodegradable substances. Chitosan films have been shown to fully degrade after just two weeks in soil, while silk fibroin films have been shown to lose 50% of their mass after four weeks [84], [85]. This discrepancy in degradation rates may also explain why the degradation rate of Shrilk was nonlinear: in the first two weeks, degradation of both chitosan and silk fibroin contributed to mass loss, whereas in the latter two weeks, most of the chitosan had already degraded and only silk fibroin degradation contributed to the overall mass loss. Regardless, this experiment confirmed that Shrilk films degrade readily in simulated landfill conditions. Any future modifications to the Shrilk manufacturing process should endeavor to maintain the film's biodegradability, as it is a crucial differentiating factor between Shrilk and conventional plastics.

3. Investigation into Silk Fibroin Substitutes

3.1 Drawbacks of Silk Fibroin

Although silk fibroin possesses unique mechanical and chemical properties, its cost, availability, and processing method make it less than ideal for mass manufacturing purposes. Compared to other natural polymers, silk is quite energy and labor intensive to produce. First of all, raising *B. mori* silkworms until they spin cocoons requires farmers to grow a sufficient amount of mulberry trees. It is estimated that silkworms consume 30-40 kg of mulberry leaves in order to produce 1 kg of raw silk [86]. The cocoons must then be heated to kill the silkworms, boiled to remove the sericin, and mechanically reeled into fibers. Taken all together, growing silkworms and harvesting silk fibers incurs significant water, energy, and labor requirements. As a result, silk is quite an expensive and limited material. Less than 100,000 metric tonnes of silk were produced globally in 2020 [87]. That same year, the price of silk cocoons in China—the number one silk producing country in the world—was 1763.85 yuan for 50kg, or roughly $5/kg [87]. By comparison, in 2019, the average market price of plastic production was roughly $1/kg [88]. In other words, plastics cost one-fifth as much as silk, and plastic production outsized silk production by nearly two orders of magnitude. Evidently, replacing plastic with silk would be economically prohibitive and practically impossible.

Furthermore, the energy and labor requirements of silk production result in a higher environmental impact. Life cycle analyses for raw silk compared to other natural fibers suggest a global warming potential over one hundred years (GWP_{100}) of 51.5 kg CO_2/kg; by comparison, the GWP_{100} of cotton fibers is only 3.4 kg CO_2/kg, and the GWP_{100} of sheep wool is only 18.5 kg CO_2/kg [89]. 28% of silk's GWP_{100} resulted from greenhouse gases emitted from the fertilized mulberry field, 18% from cocoon drying, and 17% from composting [89]. Notably, though, even

though silk has a higher environmental impact than other natural fibers, its GWP_{100} is still lower than that of plastic products. Life cycle analyses of a mixed-plastic product indicate GWP_{100} values of 119-130 kg CO_2/kg, or over double the GWP_{100} of raw silk [90].

Finally, converting raw silk into an aqueous solution which can be cast into films requires even more effort. The most common methodology for solubilizing silk dictates dissolving silk fibers in 9.3M LiBr, and then dialyzing against clean water with constant water replacements over the course of 48 hours [73]. Lithium bromide is categorized as acutely toxic when ingested orally, as well as irritating to the skin and eyes upon contact [91]. Dialysis therefore yields a significant amount of hazardous wastewater, which would be unsustainable if scaled up to mass production.

3.2 Silk Fibroin Substitutes of Interest

Taking all of these factors into account, it is important to determine whether other proteins could function similarly to silk fibroin when combined with chitosan in laminate form. A couple of factors were relevant when deciding upon potential silk fibroin substitutes. Firstly, proteins that are affordable and widely available were prioritized, because they would be able to serve as a cost-effective input for scaled-up production of Shrilk. Additionally, given that silk fibroin's ability to form β-sheet structure may be a major contributor to Shrilk's heightened tensile properties, protein candidates with distinct secondary structures were chosen so that the effect of secondary structure could be investigated. Given these considerations, three potential silk fibroin substitutes emerged: casein, collagen, and keratin.

Casein is a phosphoprotein which makes up approximately 80% of the total protein content in bovine milk [92]. It precipitates out of solution when unheated milk is acidified to pH < 4.6, making it an easily scalable protein for mass manufacturing [93]. In fact, casein is already

widely sold as a dietary supplement; in 2023, the global casein market was valued at $3.7 billion [94]. Because casein is rich in proline, which can interrupt the formation of α-helices and β-sheets, it has little secondary and tertiary structure and mostly exists as an unfolded protein [93]. All in all, casein is an accessible and affordable protein with proven mass manufacturability, as well as a random and disordered structure. These characteristics made it an interesting protein to investigate in the context of Shrilk.

Collagen is one of the most common structural proteins found in all animals. Although 28 different kinds of collagen exist in the vertebrate family alone, they are all characterized by a similar structure: three parallel peptide strands each made of three repeating amino acids, coiled around each other to form a right-handed triple helix [95]. This helical structure lends collagen thermal stability and mechanical strength. Demand for collagen has steadily increased due to its use in industries such as food and beverage, cosmetics, biomedical devices, and more. In 2023, the global collagen market was valued at $9.76 billion [96]. Appealingly, collagen supplements are produced from food industry byproducts like the skin, bones, and cartilage of cows, pigs, and fish [97]. Therefore, collagen represented another accessible and affordable protein that could serve as an alternative to silk fibroin. Unlike casein, though, collagen is a highly ordered protein, rich in helical structures.

Finally, keratin is another structural protein important to organisms across the animal kingdom. Insoluble and fibrous, keratinous proteins produce tough materials that make up many animals' epidermal appendages, including hair, nails, horns, and feathers [98]. Keratins can be classified into α-keratin, β-keratin, and γ-keratin, with α-keratin organized into α-helices, β-keratin into β-sheets, and γ-keratin into globules [99]. In this way, keratin is structurally similar to silk fibroin, which has regions of helices, sheets, and disorder. The keratin market, valued at

$1.9 billion in 2022, is the least developed of the three alternative proteins considered in this section [100]. Despite that, the growing demand for keratin driven by the cosmetics industry, as well as keratin's similarity to silk fibroin, made it an intriguing substitute protein.

3.3 Methods

Casein Films

Bovine casein (Sigma Aldrich, USA) was dissolved at 10% w/v in Milli-Q water and stirred until homogeneous. 1M NaOH was used to adjust the solution's pH to 8. The solution was stirred for another hour and then centrifuged. 20 mL of the supernatant was cast into glass molds and allowed to evaporate overnight at 50°C.

Collagen Films

Marine collagen (True Nutrition, USA) was dissolved at 10% w/v in Milli-Q water and stirred until homogeneous. 10 mL of the solution was cast into glass molds and allowed to evaporate overnight at 50°C.

Keratin Films

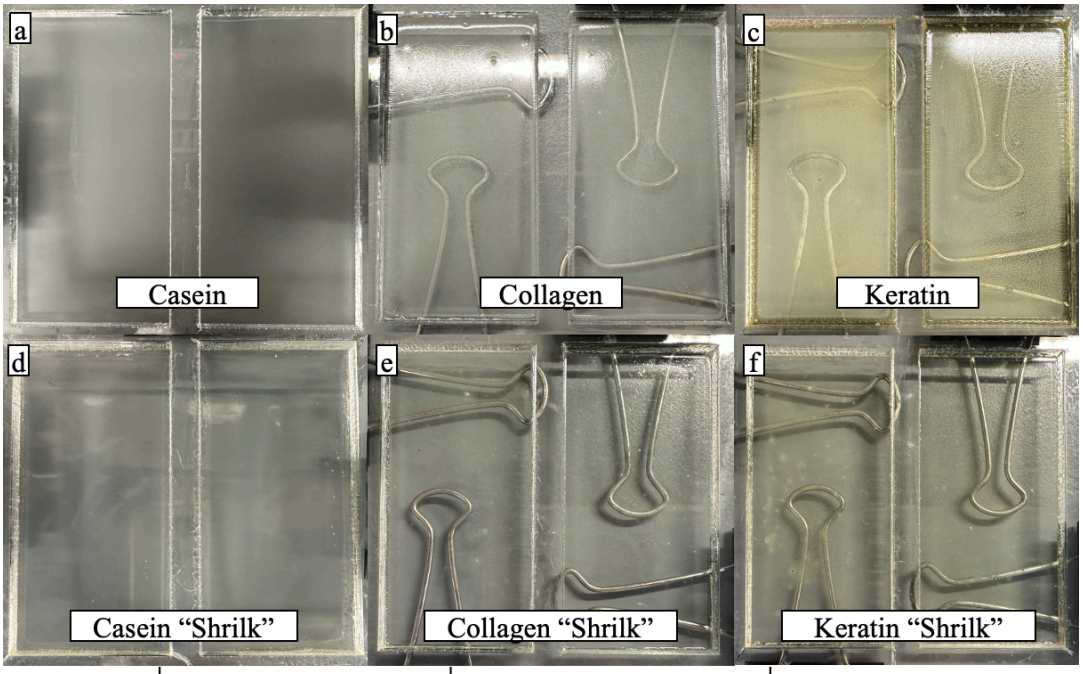
Keratin (MYOC, USA) was dissolved at 10% w/v in Milli-Q water and stirred until homogeneous. 10 mL of the solution was cast into glass molds and allowed to evaporate overnight at 50°C.

Alternative "Shrilk" Films

First, chitosan films were cast and neutralized according to the standard Shrilk protocol. Then, the substitute protein solutions were cast on top of the chitosan and dried as described in their respective sections above. Unlike silk fibroin films, alternative proteins were not washed with ethanol.

3.4 Results

All three substitute proteins were able to form a film on their own. However, none of the alternative protein films displayed the optical clarity of silk fibroin films. The casein became uniformly matte white, similar in appearance to frosted glass **(Figure 3.1a)**. The collagen, while clear in some areas, displayed microscopic cracks which gave the overall film a more opaque



g

	Casein	**Collagen**	**Keratin**
Film	• White • Matte • Uniformly smooth	• White • Small cracks throughout	• Yellow • Small cracks throughout
"Shrilk"	• Slightly cloudy • Peeled easily off the plate • delaminated after more handling	• Clear, with small cracks throughout • Delaminated immediately upon handling	• Patches of cloudiness • Brittle • Came off the glass mold in shards

Figure 3.1: Summary of silk fibroin substitute films. Top row: images of **(a)** Casein, **(b)** collagen, and **(c)** keratin films. Bottom row: images of alternative "Shrilk" films made of a chitosan layer beneath a **(d)** casein, **(e)** collagen, or **(f)** keratin layer. **(g)** Table containing an overview of alternative protein films' appearance and performance.

appearance **(Figure 3.1b)**. The keratin also displayed these microscopic cracks, and moreover had a yellowish hue **(Figure 3.1c)**. In general, the films made from substitute proteins were difficult to demold. The casein film peeled off the glass plate fairly easily, but the collagen and keratin films were extremely brittle and did not hold together well **(Figure 3.1g)**. The collagen scraped off the plate in microscopic pieces, effectively returning to a powdery state, whereas the keratin came off the plate in small shards less than one centimeter in width **(Figure 3.1g)**.

The alternative "Shrilk" films made using chitosan and silk fibroin substitutes also had limited success. Interestingly, they generally seemed more optically clear than the simple alternative protein films **(Figures 3.1d-f)**. However, neither casein nor collagen demonstrated strong adhesion to the underlying chitosan layer, and both "Shrilk" films easily delaminated upon touch (i.e. the top layer flaked off the bottom layer). The keratin seemed to adhere to the chitosan; nevertheless, like the simple keratin film, the keratin "Shrilk" was extremely brittle and broke into small shards during the demolding process. Ultimately, none of the alternative "Shrilk" films could be prepared for tensile testing **(Figure 3.1g)**.

3.5 Discussion

In conclusion, preliminary testing did not yield any proteins that could successfully serve as substitutes for silk fibroin in Shrilk. None of the three candidates—casein, collagen, and keratin—displayed the characteristics necessary to constitute a packaging material. First of all, they were not optically clear. Clarity is an essential property of Shrilk, as it is important for packaging materials to be transparent enough that consumers can see the product inside. The three alternative proteins also did not produce films with enough flexibility to be manipulated or handled. In particular, the keratin and collagen films were too brittle to even remove from the

mold. Finally, both the casein and collagen "Shrilk" films easily delaminated, suggesting that the substitute proteins were unable to create strong adhesion to the chitosan layer underneath.

Importantly, the inability to produce alternative "Shrilk" films here does not necessarily suggest that doing so is impossible. Due to time constraints, it was impossible to experiment with different processing methods and lamination techniques. It is plausible that casein, collagen, and/or keratin could form an alternative "Shrilk" film successfully if the methodology were altered—for instance, treating the chitosan with a solvent besides NaOH, or somehow modifying the substitute protein to alter its structural properties in the same way that silk fibroin is treated with methanol. Adding a crosslinker or plasticizer might improve the alternative "Shrilk" films' properties by making them stronger or less brittle. Lastly, it could be interesting to explore using combinations of proteins, especially since the procuticle after which Shrilk is modeled can contain up to hundreds of cuticular proteins [101]. For instance, resilin is a highly elastic protein commonly found in insect cuticles, which may impart some flexibility when combined with a brittle protein like keratin [102]. The rest of this thesis continues using chitosan-silk fibroin laminates, but future work should continue searching for proteins that could be used in place of silk fibroin, as making such a substitution could drastically improve Shrilk's environmental impact and cost of production.

Furthermore, given that alternative protein candidates were unsuccessful, it is important to think about ways to make silk fibroin more environmentally friendly. Since one of the main causes of greenhouse gas emissions is fertilization of the mulberry field, optimizing fertilizer application and organic waste processing can help to decrease the environmental impact associated with silk production [89]. Life cycle analyses of silkworm farming indicate that the energy used to irrigate fields, transport materials, dry silk cocoons, and more contribute

significantly to silk's overall global warming potential [89]. Encouraging farmers to utilize renewable sources of energy and giving them the resources to make the transition could therefore decrease silk's climate impact. Lastly, silkworm farming can be made more efficient by repurposing waste byproducts. For instance, silkworm pupae are often discarded, but utilizing them as a nutritious substitute for animal feed could decrease silk's GWP_{100} [103].

The environmental impact from processing raw silk into an aqueous solution could also be improved. While dissolving silk in LiBr is a well-known methodology, other solvents have been shown to break down silk fibroin as well. For instance, $CaCl_2$-H_2O is another popular solvent for silk fibroin, which is much less toxic than LiBr [104]. However, it is important to note that these different dissolution methods have been shown to impact the functionality of the resulting silk protein, due to varying polypeptide chain lengths and secondary structures [72]. Therefore, it is not necessarily the case that silk—and therefore Shrilk—made using another solvent will have the same mechanical properties as silk made using LiBr.

Finally, bioengineered silk proteins can be considered as alternatives to natural silk fibers. Recent advancements in synthetic biology have enabled scientists to develop recombinant spider silk proteins, using genetically modified bacteria, yeast, and plants to produce protein powders that can be formed into films, sponges, foams, and more [105]. (Most of this work has focused on spider silk instead of silkworm silk, since spiders are more difficult than silkworms to farm at large scale.) Because yeast and bacteria are less costly to grow than silkworms, the synthetic silks should be substantially more environmentally-friendly and inexpensive than natural silk. Much work remains to be done in order to fully replicate the original silk protein; yet it is worth noting that synthetic silks may soon be able to serve as a scalable replacement for natural silk fibers in materials like Shrilk.

4. Optimization of Material Characteristics

4.1 Industry Benchmarks

One of the reasons why plastic materials are so widely used is because of their immense range in strength and elasticity. Generally, there exists a tradeoff between tensile strength and elasticity; as tensile strength increases, elongation at break decreases. Nevertheless, even weaker plastic materials are significantly stronger than most biopolymer films. Ultimately, while the exact balance of tensile strength and plasticity may vary from material to material, plastic films tend to possess an appealing combination of both. Furthermore, plastic films have good water barrier capabilities, allowing them to retain their mechanical properties even after exposure to water [106].

In order to market Shrilk as a viable alternative to conventional plastic packaging, it is essential for Shrilk to hit the same material benchmarks set by plastic materials currently on the market. While the results in Chapter 2 demonstrated that the laminate was indeed significantly stronger than either chitosan or silk on their own, the tensile strength, toughness, and elongation displayed by the Shrilk films could still stand to be improved. Also, one of the most valuable characteristics of plastic materials is how diverse and versatile they are; thus it is desirable for Shrilk to be able to take on a wide range of mechanical properties. Therefore, this section aims to build upon previous results and provide ways to tune Shrilk's properties in order to suit a more diverse set of purposes. To that end, several experiments were conducted with the aim of increasing Shrilk's tensile strength and toughness: varying the molecular weight of chitosan, experimenting with the thicknesses of chitosan and silk fibroin layers, increasing the number of stacked laminates, and subjecting the laminates to tension. In an attempt to increase Shrilk's toughness and elongation at break, glycerol was introduced as a plasticizer for the chitosan

solution. Finally, although the Shrilk films are treated with NaOH and methanol to increase their hydrophobicity, they are still highly susceptible to wetting, and lose their mechanical properties when wet. To address this concern, Shrilk films were coated with a hydrophobic barrier and then tested under dry and wet conditions.

4.2 Methods

Shrilk Films of Different Molecular Weights

Low, medium, and high molecular weight chitosan (Sigma Aldrich, USA) was dissolved at 1% w/v in 1% v/v acetic acid and stirred until homogenous. Chitosan films were prepared using these different solutions as described previously. The rest of the protocol was carried out as previously described.

Shrilk Films of Different Thicknesses

A medium molecular weight chitosan solution was prepared as previously described. Instead of 6.75 mL, as the original method calls for, 4 mL, 7 mL, and 10 mL of chitosan solution were cast onto glass plates instead. After drying, a layer of silk fibroin was cast on top of the chitosan such that the ratio of chitosan to silk fibroin solids was 1:2. The rest of the protocol was carried out as previously described.

Multilayered Shrilk Films

Shrilk films were fabricated as described previously. Prior to layering, dried films were submerged in Milli-Q water for 1 minute to rehydrate. The first film was placed on a glass plate, chitosan-side down, then the next layer was placed on top such that the silk side of the first film faced the chitosan side of the second. Films were smoothed out using a finger to remove any wrinkles and/or trapped air bubbles, and blotted to remove excess water. The process was repeated as many times as necessary to obtain the desired number of layers. The multilayered

films were allowed to dry and naturally peel off the glass plate.

Shrilk Films Incorporating Glycerol

Medium molecular weight chitosan (Sigma Aldrich, USA) was dissolved at 1% w/v in 1% v/v acetic acid and stirred until homogenous. Glycerol was added into the chitosan solution at 5%, 10%, and 15% w/w respective to the dry weight of chitosan. The solution was stirred for one hour to ensure homogeneity. Plasticized chitosan films were then fabricated according to the standard protocol. For half of the films, a silk fibroin layer was cast on top of the chitosan as usual. For the other half, silk fibroin mixed with 5%, 10%, and 15% w/w respective to the dry weight of silk fibroin and cast on top of the chitosan, such that both layers had the same ratio of glycerol to biopolymer. All films were washed with methanol and allowed to dry.

Chitosan Films With and Without NaOH Treatment

Medium molecular weight chitosan (Sigma Aldrich, USA) was dissolved at 1% w/v in 1% v/v acetic acid and stirred until homogenous. Glycerol was added into the chitosan solution at 50% w/w respective to the dry weight of chitosan. The solution was stirred for one hour to ensure homogeneity. Plasticized chitosan films were cast and dried at 50°C overnight. Half of the films were directly unmolded; the other half were submerged in 1M NaOH for 10 minutes and washed with Milli-Q water. Films were allowed to dry.

Shrilk Films with Hydrophobic Coating

Shrilk films were prepared as previously described. Wax-It-All Food-Grade Wax (Howard Products, USA) and Heavy Duty Fabric Wax (Otter Wax, USA) were applied to the surface of Shrilk films according to manufacturer instructions. The coated Shrilk films were allowed to sit for 24 hours at room temperature, and then cut into 1.5 cm x 5 cm rectangles for tensile testing.

Wet Tensile Testing

Control Shrilk films and Shrilk films covered in hydrophobic coatings were submerged in water for one minute, and then blotted to remove excess water. Stress-strain measurements were taken as previously described.

Contact Angle Measurement

To create a sessile droplet, 0.2 mL drops of Milli-Q water were dispensed from a 1 mL syringe onto the film surface. Contact angle images were taken using the Droplet Lab Tensiometer (Droplet Lab, Canada). Images were loaded into ImageJ and analyzed using the DropSnake plugin [107].

4.3 Results

Three sets of alterations were made to Shrilk films in an attempt to increase their tensile strength and toughness: using chitosan of varying molecular weights, making Shrilk films with different thicknesses, and stacking several Shrilk films on top of each other. In general, none of these three modifications were found to have a significant effect on Shrilk film properties.

One-way ANOVA yielded no significant differences in tensile strength ($p = 0.242$), toughness ($p = 0.373$), or elongation at break ($p = 0.117$) as a result of changing the molecular weight of the chitosan layer **(Figure 4.1a-c)**. Interestingly, though, the variance in tensile strength associated with low molecular weight Shrilk films was significantly smaller than that of medium molecular weight films ($p = 0.011$) and high molecular weight films ($p = 0.050$) **(Figure 4.1a)**. Similarly, changing the thickness of the Shrilk film had no significant effect on tensile strength ($p = 0.972$), toughness ($p = 0.396$), or elongation at break ($p = 0.179$) **(Figure 4.1d-f)**. Finally, increasing the number of layers had no significant effect on tensile strength ($p = 0.629$), toughness ($p = 0.507$), or elongation at break ($p = 0.163$). Notably, one-way ANOVA did reveal a

highly significant difference between the mean tensile strength ($p < 0.001$), toughness ($p < 0.001$), and elongation at break ($p = 0.003$) **(Figure 4.1g-i)** of different experimental conditions.

Following these experiments which attempted to increase the tensile strength and toughness of Shrilk films, glycerol was used as a plasticizer to try to increase the films' elongation at break values. When glycerol was only added to the chitosan layer, no significant

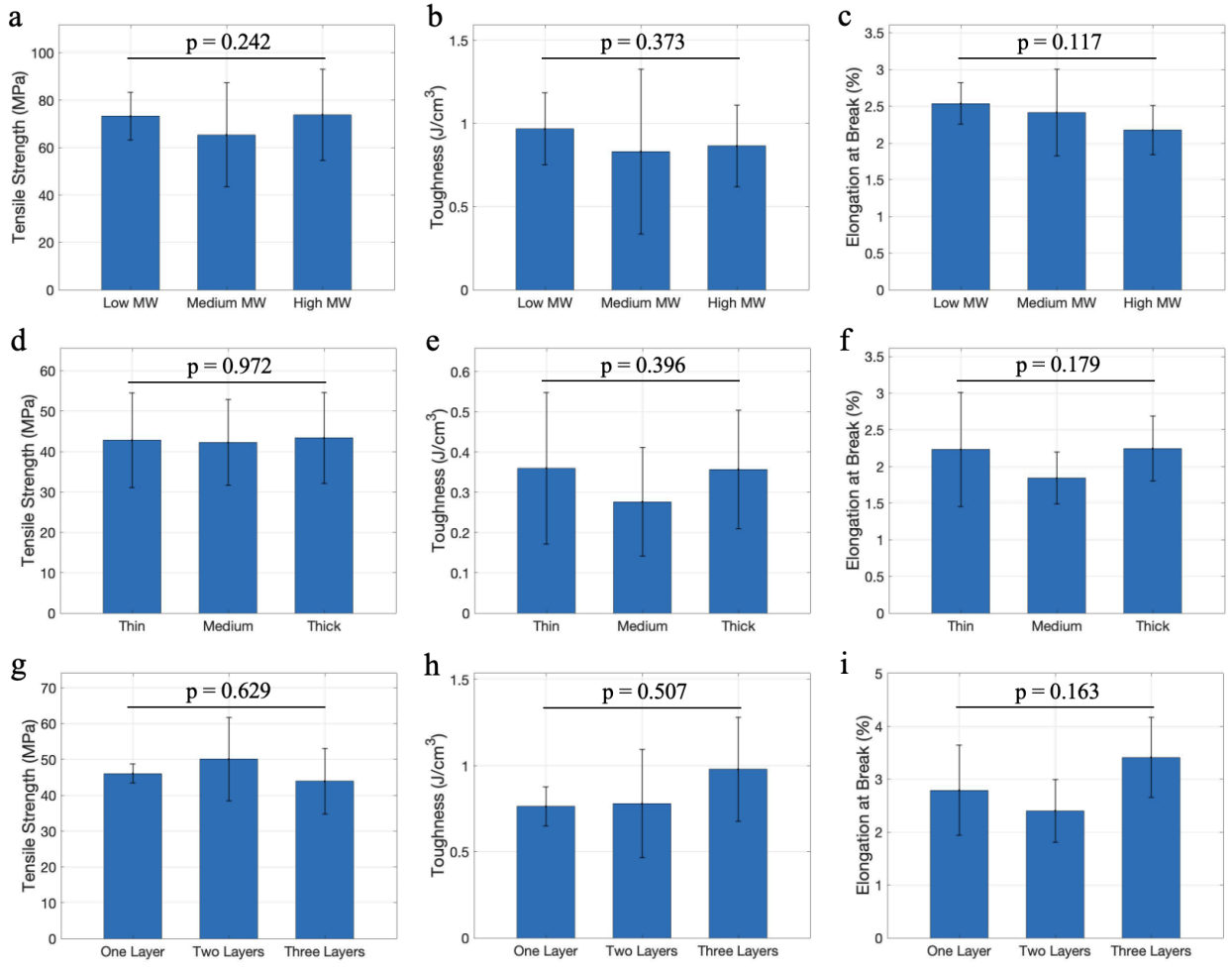


Figure 4.1: Tensile properties of altered Shrilk films. (a) Tensile strength, **(b)** toughness, and **(c)** elongation at break for Shrilk films made with low, medium, and high molecular weight chitosan. **(d)** Tensile strength, **(e)** toughness, and **(f)** elongation at break for thin, medium, and thick Shrilk films. **(g)** Tensile strength, **(h)** toughness, and **(i)** elongation at break for multi-layer Shrilk films made with one, two, and three layers. Mean values are shown and error bars represent ± standard deviations (n = 3-6 samples per group), as analyzed using a one-way ANOVA test.

effect was observed on Shrilk's tensile strength (p = 0.593), toughness (p = 0.635), or elongation at break (p = 0.776) **(Figure 4.2a-c)**. However, when glycerol was added to both chitosan and silk layers, one-way ANOVA yielded significant differences in tensile strength (p = 0.002), toughness (p = 0.009), and elongation at break (p = 0.008) **(Figure 4.2a-c)**. As the percentage of plasticizer increased from 5% to 15%, mean tensile strength decreased by 55% (p = 0.012) and toughness decreased by 74% (p = 0.002). Somewhat surprisingly, elongation at break also decreased by 62% (p = 0.004). In general, the elongation at break values after adding glycerol were consistently within 1-3%, similar to those of unplasticized Shrilk films.

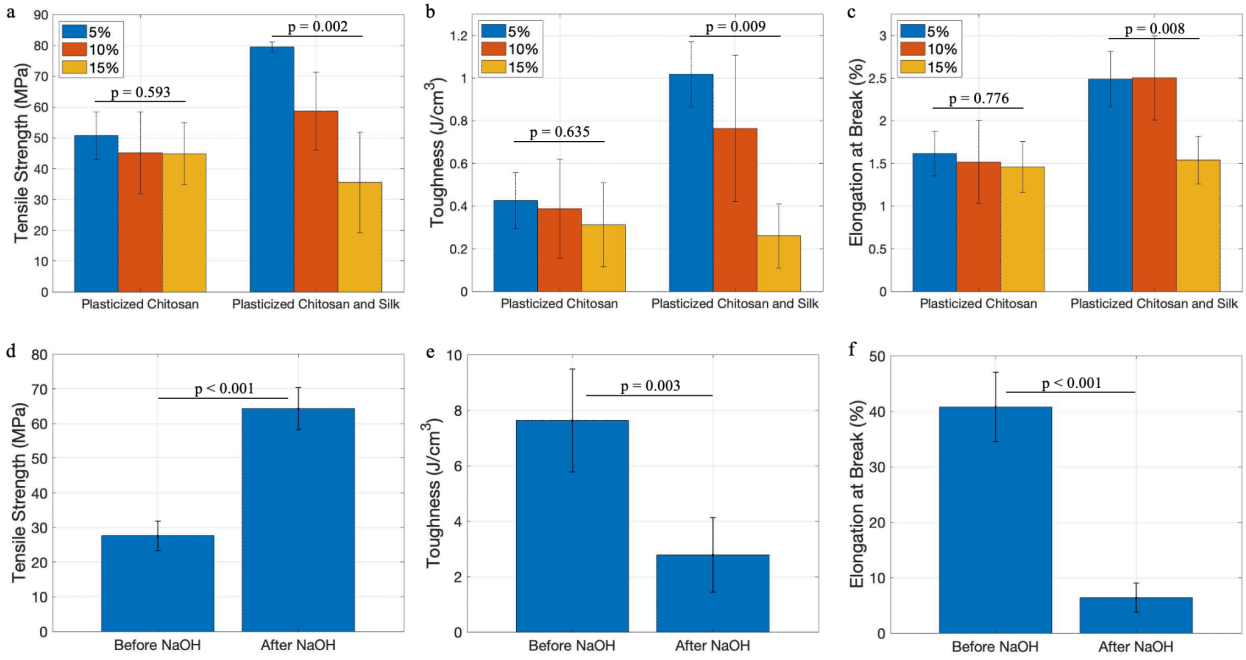


Figure 4.2: Tensile properties of plasticized Shrilk and chitosan films. (a) Tensile strength, **(b)** toughness, and **(c)** elongation at break for Shrilk films with plasticized chitosan, as well as plasticized chitosan chitosan and silk. **(d)** Tensile strength, **(e)** toughness, and **(f)** elongation at break for plasticized chitosan films before and after NaOH treatment. Mean values are shown and error bars represent ± standard deviations (n = 4-6 samples per group). Groupwise p-values **(a-c)** were calculated using a one-way ANOVA test; pairwise p-values **(d-f)** were calculated using two-tailed Student's T-tests.

In order to understand why higher levels of plasticizer were not resulting in larger elongation at break values, the effect of NaOH treatment was investigated. Tensile testing revealed that washing chitosan films containing 50% w/w glycerol with 1M NaOH more than doubled their tensile strength ($p < 0.001$), decreased toughness by a factor of 2.5 ($p = 0.003$), and decreased elongation at break by a factor of six, from 32% to 5% ($p < 0.001$) **(Figure 4.2d-f)**. Since the chitosan layers in Shrilk films are washed with NaOH as part of the fabrication process, these results help explain why glycerol was not effective in increasing the plasticity of Shrilk films.

Finally, Shrilk films were covered in hydrophobic coatings to improve their resistance to water. Applying Wax-It-All (a blend of beeswax, carnauba wax, and food grade mineral oil) increased the contact angle of the Shrilk film by 35% as compared to the untreated chitosan layer ($p = 0.005$) and 50% as compared to the untreated silk layer ($p = 0.001$) **(Figure 4.3a, b)**. Otter Wax (a blend of beeswax, plant-based waxes, and plant-based oils) increased the contact angle of the Shrilk film by 21% as compared to the untreated chitosan layer ($p = 0.051$) and 34% as compared to the untreated silk layer ($p = 0.014$) **(Figure 4.3a, b)**. The mean contact angle measured for Wax-It-All-treated films was higher than that of Otter Wax-treated films (81° vs 73°, respectively), but the difference was not statistically significant ($p = 0.224$).

The effect of hydrophobic coatings on contact angles was corroborated by their effect on tensile properties. Whereas the tensile strength of untreated Shrilk films decreased by more than a factor of 10 when wet ($p < 0.001$), the Wax-It-All-treated and Otter Wax-treated films' tensile strengths only decreased by a factor of 2.46 ($p = 0.024$) and 2.89 ($p = 0.008$), respectively **(Figure 4.3c)**. Furthermore, the untreated films experienced no significant change in toughness when wet ($p = 0.324$), whereas treated films more than doubled in toughness when wet, although

the effect was only statistically significant for Otter Wax ($p = 0.013$) and slightly over the p-value threshold for Wax-It-All ($p = 0.082$) **(Figure 4.3d)**. All three groups experienced a 4-6x increase in elongation at break when wet (untreated: $p = 0.008$; Wax-It-All: $p = 0.046$; Otter Wax: $p = 0.040$) **(Figure 4.3e)**. However, one-way ANOVA revealed no significant difference in elongation at break within the three experimental groups ($p = 0.437$) **(Figure 4.3e)**.

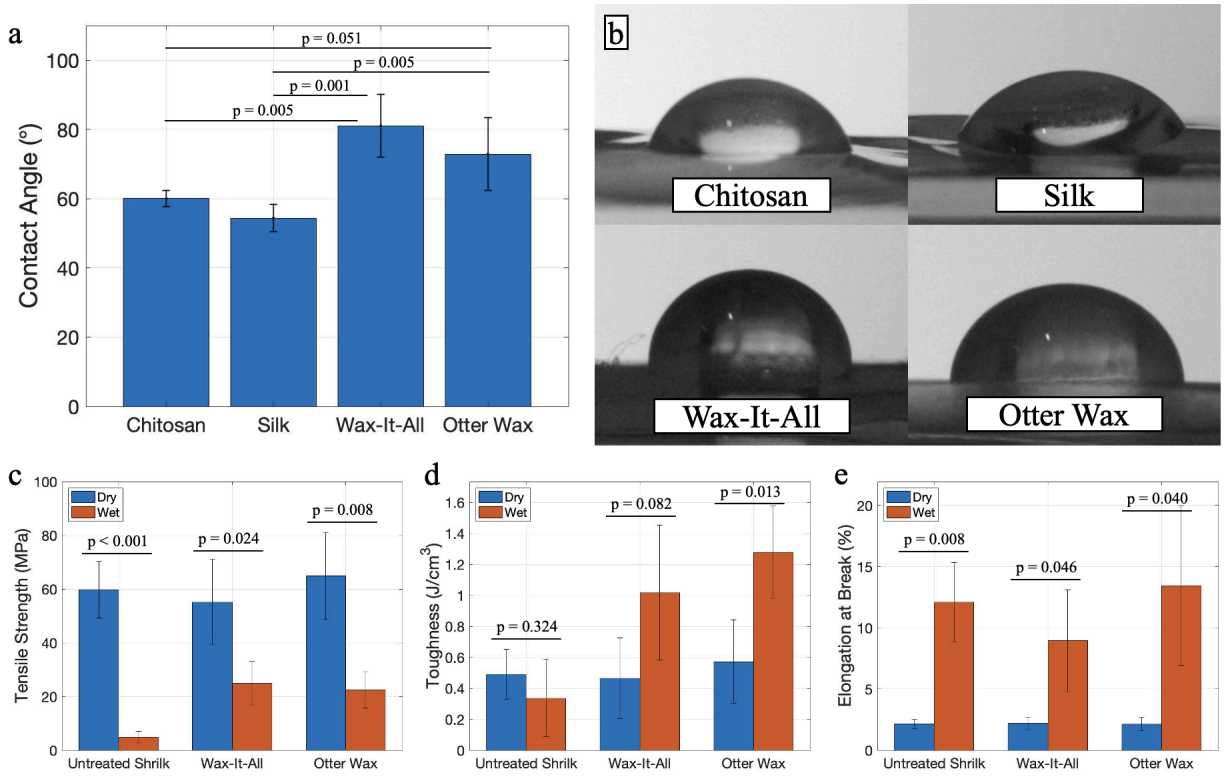


Figure 4.3: Effects of hydrophobic coatings on Shrilk. (a, b) Hydrophobic coatings significantly increased the contact angle of water droplets on Shrilk. **(c)** When hydrophobic coatings were applied, wet Shrilk pieces maintained more of their dry tensile strength than untreated samples. On the other hand, **(d)** toughness and **(e)** elongation at break were higher for wet Shrilk pieces than dry ones. Mean values are shown and error bars represent ± standard deviations (n = 4-6 samples per group). Pairwise p-values were calculated using two-tailed Student's T-tests.

4.4 Discussion

The experiments comprising this chapter had three main objectives: to increase the tensile strength and toughness of Shrilk films, to increase the elongation at break of Shrilk films, and to

maintain Shrilk films' tensile properties even after coming into contact with water.

Ultimately, none of the three experiments aimed at increasing tensile strength or toughness led to any significant improvements. These findings were somewhat surprising, yet illuminating. High molecular weight chitosan has been shown to have superior tensile strength to low molecular weight chitosan films, as the longer strands allow for greater chain entanglement and thus stronger mechanical properties [108]. The lack of response to different molecular weight chitosan may indicate that the strength of the chitosan layer itself is not the dominant factor in determining the tensile strength of the Shrilk film as a whole. On the other hand, the low molecular weight Shrilk films had significantly lower variation in tensile strength than the medium and high molecular weight films. When making the films, the low molecular weight chitosan dissolved easily into 1% v/v acetic acid, whereas the medium and high molecular weight chitosan solutions had small pieces of chitosan that were not fully dissolved even after stirring overnight. These irregularities may have caused heterogeneities in the films, increasing the variance of tensile strengths. Given that the molecular weight did not seem to have an effect on Shrilk's tensile strength, it may be better to use low molecular weight chitosan in the future, in order to minimize batch-to-batch variation and create a more uniform product.

Varying the thickness of the Shrilk films was intended to provide insight on the importance of the chitosan-silk fibroin interface for imparting tensile strength and toughness. It was hypothesized that the thinner films would have superior mechanical properties, as the interface made up a larger proportion of the film. However, the thickness of the films did not make any difference on the films' properties. This might indicate that the interface is also not the major contributor to Shrilk's mechanical properties; on the other hand, it might suggest that the width of the interface is proportional to the thickness of the films. For instance, since the thicker

films require more silk fibroin solution to be cast on top of the chitosan, they may take longer to dry, giving the silk fibroin more time to penetrate into the chitosan layer. Nonetheless, the fact that Shrilk films' tensile properties are independent of thickness is not necessarily a drawback. In fact, because film thickness does not seem to matter, manufacturers could produce Shrilk films of a range of thicknesses without having to worry about adverse consequences. Of course, the maximum film thickness used in the conducted experiment was approximately 150 μm, so tensile properties for much thicker films should not be extrapolated from these findings. Nevertheless, the obtained results provide manufacturers with a range of thicknesses (i.e. that could simplify production and save time, space, and money.

In a similar vein, experimentation also revealed that the number of layers in multi-layer Shrilk films did not significantly impact tensile properties. In other words, the maximum stress that Shrilk films could withstand increased linearly with the number of layers stacked on top of each other. It was hypothesized that tensile strength would increase with the number of layers, similar to how overlapping regions of nonuniformly-oriented planes give strength to the exoskeleton [44]. However, this hypothesis did not hold. Nonetheless, this finding suggests that manufacturers could easily scale up the number of layers used in a given film in order to make it withstand a desired amount of stress. Moreover, the films were bonded together using only water, without the need for additional adhesives. This simplification makes the stacking process relatively facile and cost-effective. Lastly, while the thickness experiments described above should not be extrapolated to films that are much thicker than 150 μm, the stacking method is likely to be consistently successful even for thicker films, as it does not alter the films' inherent properties in any way but simply sticks multiple films together.

After attempting to increase the tensile strength and toughness of Shrilk films, glycerol was added in an effort to plasticize the Shrilk films. Glycerol is a commonly used plasticizer for both chitosan and silk films; the glycerol molecules interrupt hydrogen bonding between chitosan and silk chains, typically resulting in films that are weaker but more flexible and plastic [109], [110]. As expected, tensile strength and toughness decreased significantly as the percentage of glycerol increased. However, the elongation at break values stayed quite low, within 1-3%. Subsequent experiments demonstrated that NaOH treatment of films containing glycerol reversed much of the plasticizing effect; tensile strength rebounded while elongation at break dropped drastically. Upon further reading, this reversal may be due to glycerol's solubility properties. The glycerol molecule is a short hydrocarbon containing three -OH groups; this structure, which enables it to form hydrogen bonds and have a plasticizing effect on chitosan and silk, also makes it highly soluble in polar solvents such as NaOH and methanol [111], [112]. When the films were treated with NaOH and methanol as the Shrilk protocol demands, some of the glycerol may have been solubilized and pulled out of the film, causing the plasticizing effect to be lost. Additional plasticizer experiments could not be performed due to time constraints, but future experiments should utilize plasticizers that are not soluble in polar solvents. For instance, epoxidized soybean oil and epoxidized palm oil are commercially-available, eco-friendly plasticizers that may be less soluble in polar solvents due to their long hydrocarbon chains [113]. It is essential to find a plasticizer that can withstand NaOH and methanol treatments, as they are key parts of Shrilk fabrication: the NaOH deprotonates the chitosan layer to allow bonding to silk fibroin, and the methanol encourages silk fibroin to form ordered β-sheets.

Perhaps the most interesting set of experiments was coating Shrilk films in hydrophobic waxes to improve their water resistance. Although Wax-It-All and Otter Wax treatment were not

able to fully maintain Shrilk's tensile properties after wetting, they did reduce the drop in tensile strength significantly. On the other hand, the toughness and elongation at break of waxed films actually increased in their wet state. These findings indicate that water could actually act as a plasticizer in slightly hydrated films, much like the way that glycerol had been intended to act as a plasticizer. Whereas the untreated Shrilk film had extremely low tensile strength when wet, the treated films only became slightly hydrated and maintained decent tensile strength but were able to stretch more and absorb more energy before rupturing—both important characteristics for plastic alternatives. In fact, water content has been shown to play an important role in modulating the mechanical properties of insect cuticles, suggesting that insects might even use cuticular transpiration to regulate their own strength and toughness [114].

These results indicate that the amount of water contained in Shrilk films has a critical effect on their tensile properties, which may explain some of the batch-to-batch variation that was observed throughout this study. Inconsistencies in humidity, both in the room and in the oven used to dry the films overnight, may have resulted in Shrilk films of varying levels of hydration, and thus varying tensile strengths and toughnesses. Future experiments should investigate the effect of humidity on Shrilk films and control for humidity when making films going forward. Additionally, Wax-It-All and Otter Wax only served as a proof-of-concept for the idea of treating Shrilk films to make them more water-resistant. Future experiments should also search for treatments which impart even higher hydrophobicity. After all, the contact angles for both Wax-It-All and Otter Wax-treated films were still under 90°, meaning that the treated Shrilk films were still technically hydrophilic surfaces according to American Chemical Society standards [115]. However, since biodegradability is one of Shrilk's key features, it is important that whatever water-resistant treatment chosen for Shrilk retains the films' biodegradability.

Wax-It-All and Otter Wax are both made with natural waxes and oils, making them inherently biodegradable substances. Although traditional water-resistant materials are typically made from petroleum products like polymethyl methacrylate and polyurethane, a new wave of eco-friendly waterproofing treatments using natural materials and sustainable practices has also arisen, which Shrilk should draw upon [116].

5. Conclusion

The work performed in this study presents a compelling argument for further research and development into the concept of chitosan-protein laminates as a potential alternative to conventional, petroleum-based plastic packaging materials. Chitosan-silk fibroin films (referred to in this study as "Shrilk") were successfully reproduced; they demonstrated optical clarity and a higher tensile strength than either chitosan or silk on their own, as well as a homogeneously blended film. In fact, the tensile strength of these Shrilk films was comparable to some plastic materials currently on the market, such as polystyrene. Although the mechanism behind this heightened strength is still somewhat unclear, FTIR and Raman spectroscopy suggest that this effect may be due to organized mechanical entanglement of chitosan and silk fibroin strands. Importantly, unlike petroleum-based plastics, Shrilk films were readily biodegradable, losing 84% of their total mass after 4 weeks in simulated landfill conditions.

In contrast to our hypothesis, efforts to reproduce "Shrilk" using proteins besides silk fibroin were unsuccessful. However, casein, collagen, and keratin still might be able to function as substitutes for silk fibroin with the use of additional chemical crosslinkers, plasticizers, and/or post-casting treatments. Although these avenues of exploration were not in the scope of this project, future work should investigate these methods. Future research should also consider searching for other proteins with similar chemical and structural properties to silk fibroin, using combinations of multiple proteins, and investigating the environmental and cost implications of using synthetic or bioengineered silk.

Efforts to manipulate the tensile properties of Shrilk revealed that the molecular weight of chitosan, thickness of films, number of laminate layers, and addition of glycerol had little consequence. However, covering Shrilk films with a hydrophobic coating proved effective in

reducing Shrilk's vulnerability to water and even indicated that a moderate amount of water could act as a plasticizer, increasing films' toughness and elongation at break. Future research might investigate other ways to alter Shrilk's tensile properties and increase tensile strength, toughness, or elongation at break. On the other hand, Shrilk's lack of response to the factors tested in this study demonstrate the robustness of Shrilk, suggesting that its properties would not be significantly affected by small changes in the manufacturing process.

The plastic industry is a key contributor to environmental damage, from its greenhouse gas emissions to its impact on wildlife. Going forward, reducing the world's dependence on petroleum-based plastics will play a crucial part in confronting the climate crisis. This thesis provides an overview of the structure and properties of Shrilk, a biodegradable alternative to conventional plastic packaging. Shrilk is made up of environmentally friendly constituents: chitosan is sourced from seafood waste byproducts, and silk fibroin has been shown to have a lower global warming potential than fossil-fuel based plastics produced today. As a film, it demonstrates tensile properties comparable to common plastic packaging materials. It can be treated with a hydrophobic coating to maintain these tensile properties when in wet environments. It is optically clear and readily biodegradable. Thus, Shrilk represents one potential solution to the plastic pollution issue—an essential problem we must conquer if we are ever to build a sustainable future.

References

[1] "Method of making insoluble products of phenol and formaldehyde." [Online]. Available: https://patents.google.com/patent/US942699A/en

[2] R. C. Thompson, C. J. Moore, F. S. vom Saal, and S. H. Swan, "Plastics, the environment and human health: current consensus and future trends," *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, vol. 364, no. 1526, pp. 2153–2166, Jul. 2009, doi: 10.1098/rstb.2009.0053.

[3] R. Geyer, J. R. Jambeck, and K. L. Law, "Production, use, and fate of all plastics ever made," *Sci Adv*, vol. 3, no. 7, p. e1700782, Jul. 2017, doi: 10.1126/sciadv.1700782.

[4] L. Corbari, A. Maltese, F. Capodici, M. C. Mangano, G. Sarà, and G. Ciraolo, "Indoor spectroradiometric characterization of plastic litters commonly polluting the Mediterranean Sea: toward the application of multispectral imagery," *Sci. Rep.*, vol. 10, no. 1, p. 19850, Nov. 2020, doi: 10.1038/s41598-020-74543-6.

[5] Zion Market Research, "Plastic market size, trends, demand-supply, applications 2023-2030," Zion Market Research. Accessed: Jan. 10, 2024. [Online]. Available: https://www.zionmarketresearch.com/report/plastic-market

[6] L. A. Hamilton and S. Feit, "Plastic & Climate: The hidden costs of a plastic planet," 2019, Accessed: Jan. 09, 2024. [Online]. Available: https://policycommons.net/artifacts/2485040/untitled/3507468/

[7] J. Zheng and S. Suh, "Strategies to reduce the global carbon footprint of plastics," *Nat. Clim. Chang.*, vol. 9, no. 5, pp. 374–378, Apr. 2019, doi: 10.1038/s41558-019-0459-z.

[8] "Chapter 2 — Global Warming of 1.5 ºC." Accessed: Mar. 29, 2024. [Online]. Available: https://www.ipcc.ch/sr15/chapter/chapter-2/

[9] F. Vidal *et al.*, "Designing a circular carbon and plastics economy for a sustainable future," *Nature*, vol. 626, no. 7997, pp. 45–57, Feb. 2024, doi: 10.1038/s41586-023-06939-z.

[10] "Fossils, Plastics, & Petrochemical Feedstocks," Center for International Environmental Law, 2017. Accessed: Jan. 10, 2024. [Online]. Available: https://www.ciel.org/wp-content/uploads/2017/09/Fueling-Plastics-Fossils-Plastics-Petrochemical-Feedstocks.pdf

[11] C. P. Ward and C. M. Reddy, "We need better data about the environmental persistence of plastic goods," *Proceedings of the National Academy of Sciences*, vol. 117, no. 26, pp. 14618–14621, 2020, doi: 10.1073/pnas.2008009117.

[12] E. Schmaltz *et al.*, “Plastic pollution solutions: emerging technologies to prevent and collectmarineplastic pollution,” *Environ. Int.*, vol. 144, p. 106067, Nov. 2020, doi: 10.1016/j.envint.2020.106067.

[13] M. N. Basto *et al.*, “Plastic ingestion in aquatic birds in Portugal,” *Mar. Pollut. Bull.*, vol. 138, pp. 19–24, Jan. 2019, doi: 10.1016/j.marpolbul.2018.11.024.

[14] C. Wang *et al.*, “Microplastic Pollution in the Soil Environment: Characteristics, Influencing Factors, and Risks,” *Sustain. Sci. Pract. Policy*, vol. 14, no. 20, p. 13405, Oct. 2022, doi: 10.3390/su142013405.

[15] T. R. Gaspar, R. J. Chi, M. W. Parrow, and A. H. Ringwood, “Cellular Bioreactivity of Micro- and Nano-Plastic Particles in Oysters,” *Frontiers in Marine Science*, vol. 5, 2018, doi: 10.3389/fmars.2018.00345.

[16] G. Liu, Z. Zhu, Y. Yang, Y. Sun, F. Yu, and J. Ma, “Sorption behavior and mechanism of hydrophilic organic chemicals to virgin and aged microplastics in freshwater and seawater,” *Environ. Pollut.*, vol. 246, pp. 26–33, Mar. 2019, doi: 10.1016/j.envpol.2018.11.100.

[17] X. Li, R. Wang, W. Dai, Y. Luan, and J. Li, “Impacts of Micro(nano)plastics on Terrestrial Plants: Germination, Growth, and Litter,” *Plants*, vol. 12, no. 20, Oct. 2023, doi: 10.3390/plants12203554.

[18] R. Kumar *et al.*, “Micro(nano)plastics pollution and human health: How plastics can induce carcinogenesis to humans?,” *Chemosphere*, vol. 298, p. 134267, Jul. 2022, doi: 10.1016/j.chemosphere.2022.134267.

[19] M. Raffaele *et al.*, “Microplastics and Nanoplastics in Atheromas and Cardiovascular Events,” *N. Engl. J. Med.*, vol. 390, no. 10, pp. 900–910, Mar. 2024, doi: 10.1056/NEJMoa2309822.

[20] H. A. Leslie, M. J. M. van Velzen, S. H. Brandsma, A. D. Vethaak, J. J. Garcia-Vallejo, and M. H. Lamoree, “Discovery and quantification of plastic particle pollution in human blood,” *Environ. Int.*, vol. 163, p. 107199, May 2022, doi: 10.1016/j.envint.2022.107199.

[21] D. Fullerton and R. Stavins, “How economists see the environment,” Nature Publishing Group UK. Accessed: Mar. 30, 2024. [Online]. Available: http://dx.doi.org/10.1038/26606

[22] R. Lazarus, “Super wicked problems and climate change: Restraining the present to liberate the future,” *Cornell Law Rev.*, vol. 94, p. 1153, Nov. 2008, [Online]. Available: https://heinonline.org/hol-cgi-bin/get_pdf.cgi?handle=hein.journals/clqv94&section=38

[23] W. D. Nordhaus, “Some foundational and transformative grand challenges for the social

and behavioral sciences: The problem of global public goods," *SSRN Electron. J.*, 2010, doi: 10.2139/ssrn.1889357.

[24] R. Karasik *et al.*, "20 Years of Government Responses to the Global Plastic Pollution Problem," Nicholas Institute for Environmental Policy Solutions, Duke University, May 2020. Accessed: Mar. 09, 2024. [Online]. Available: https://dukespace.lib.duke.edu/items/7d74d9c7-84f9-4ba2-8392-c84c931b4cd0

[25] S. Wang, "International law-making process of combating plastic pollution: Status Quo, debates and prospects," *Mar. Policy*, vol. 147, p. 105376, Jan. 2023, doi: 10.1016/j.marpol.2022.105376.

[26] G. Ambassadors, "Historic day in the campaign to beat plastic pollution: Nations commit to develop a legally binding agreement," UN Environment. Accessed: Mar. 30, 2024. [Online]. Available: https://www.unep.org/news-and-stories/press-release/historic-day-campaign-beat-plastic-pollution-nations-commit-develop

[27] "Plastics Policy Inventory Search." Accessed: Mar. 30, 2024. [Online]. Available: https://nicholasinstitute.duke.edu/plastics-policy-inventory/search

[28] D. Xanthos and T. R. Walker, "International policies to reduce plastic marine pollution from single-use plastics (plastic bags and microbeads): A review," *Mar. Pollut. Bull.*, vol. 118, no. 1–2, pp. 17–26, May 2017, doi: 10.1016/j.marpolbul.2017.02.048.

[29] J. Dikgang and M. Visser, "Behavioural response to plastic bag legislation in Botswana," *S. Afr. J. Econ.*, vol. 80, no. 1, pp. 123–133, Mar. 2012, doi: 10.1111/j.1813-6982.2011.01289.x.

[30] L. M. Heidbreder, I. Bablok, S. Drews, and C. Menzel, "Tackling the plastic problem: A review on perceptions, behaviors, and interventions," *Sci. Total Environ.*, vol. 668, pp. 1077–1093, Jun. 2019, doi: 10.1016/j.scitotenv.2019.02.437.

[31] A. L. Andrady and M. A. Neal, "Applications and societal benefits of plastics," *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, vol. 364, no. 1526, pp. 1977–1984, Jul. 2009, doi: 10.1098/rstb.2008.0304.

[32] British Plastics Federation, "How is plastic made? A simple step-by-step explanation," British Plastics Federation. Accessed: Jan. 11, 2024. [Online]. Available: https://www.bpf.co.uk/plastipedia/how-is-plastic-made.aspx

[33] *Bio-Based Polymers: Handbook of R&D Information in JAPAN*. CMC Pub., 2013. [Online]. Available: https://play.google.com/store/books/details?id=u8twmwEACAAJ

[34] "Bio-based polymers: A green alternative to traditional plastics," Chemical Abstracts

Service, 2022. Accessed: Jan. 11, 2024. [Online]. Available: https://www.cas.org/sites/default/files/documents/%7Bd7e92e91-38ab-460b-a56a-c744f334d246%7D_CASGENENGWHP100603220503-BioBased-Polymers-WhitePaper.pdf

[35] N. F. Zaaba and M. Jaafar, "A review on degradation mechanisms of polylactic acid: Hydrolytic, photodegradative, microbial, and enzymatic degradation," *Polym. Eng. Sci.*, vol. 60, no. 9, pp. 2061–2075, Sep. 2020, doi: 10.1002/pen.25511.

[36] M. M. Abe *et al.*, "Advantages and Disadvantages of Bioplastics Production from Starch and Lignocellulosic Components," *Polymers* , vol. 13, no. 15, Jul. 2021, doi: 10.3390/polym13152484.

[37] "Overview of materials for Low Density Polyethylene (LDPE), Film Grade." Accessed: Jan. 15, 2024. [Online]. Available: https://www.matweb.com/search/DataSheet.aspx?MatGUID=9ff98d958a714b2a8a00990a929d6f14

[38] "Overview of materials for High Density Polyethylene (HDPE), Film Grade." Accessed: Jan. 15, 2024. [Online]. Available: https://www.matweb.com/search/DataSheet.aspx?MatGUID=af3d832d12b64e0ea1c0657208ae5b87

[39] T. Jiang, Q. Duan, J. Zhu, H. Liu, and L. Yu, "Starch-based biodegradable materials: Challenges and opportunities," *Advanced Industrial and Engineering Polymer Research*, vol. 3, no. 1, pp. 8–18, Jan. 2020, doi: 10.1016/j.aiepr.2019.11.003.

[40] M. Hedar, A. Intisar, T. Hussain, N. Hussain, and M. Bilal, "Challenges and Issues in Biopolymer Applications," in *Handbook of Biopolymers*, S. Thomas, A. Ar, C. Jose Chirayil, and B. Thomas, Eds., Singapore: Springer Nature Singapore, 2022, pp. 1–16. doi: 10.1007/978-981-16-6603-2_55-1.

[41] D. Zhang, E. A. del Rio-Chanona, and N. Shah, "Life cycle assessment of bio-based sustainable polylimonene carbonate production processes," in *Computer Aided Chemical Engineering*, vol. 44, M. R. Eden, M. G. Ierapetritou, and G. P. Towler, Eds., Elsevier, 2018, pp. 1693–1698. doi: 10.1016/B978-0-444-64241-7.50277-9.

[42] J. G. Fernandez and D. E. Ingber, "Unexpected strength and toughness in chitosan-fibroin laminates inspired by insect cuticle," *Adv. Mater.*, vol. 24, no. 4, pp. 480–484, Jan. 2012, doi: 10.1002/adma.201104051.

[43] J. F. V. Vincent and U. G. K. Wegst, "Design and mechanical properties of insect cuticle," *Arthropod Struct. Dev.*, vol. 33, no. 3, pp. 187–199, Jul. 2004, doi: 10.1016/j.asd.2004.05.006.

[44] H.-O. Fabritius, C. Sachs, P. R. Triguero, and D. Raabe, “Influence of structural principles on the mechanics of a biological fiber-based composite material with hierarchical organization: The exoskeleton of the LobsterHomarus americanus,” *Adv. Mater.*, vol. 21, no. 4, pp. 391–400, Jan. 2009, doi: 10.1002/adma.200801219.

[45] S. Muthukrishnan, S. Mun, M. Y. Noh, E. R. Geisbrecht, and Y. Arakane, “Insect Cuticular Chitin Contributes to Form and Function,” *Curr. Pharm. Des.*, vol. 26, no. 29, pp. 3530–3545, 2020, doi: 10.2174/1381612826666200523175409.

[46] *Global Plastics Outlook: Economic Drivers, Environmental Impacts and Policy Options*. OECD, 2022. doi: 10.1787/de747aef-en.

[47] S. Peter, N. Lyczko, D. Gopakumar, H. J. Maria, A. Nzihou, and S. Thomas, “Chitin and Chitosan Based Composites for Energy and Environmental Applications: A Review,” *Waste and Biomass Valorization*, vol. 12, no. 9, pp. 4777–4804, Sep. 2021, doi: 10.1007/s12649-020-01244-6.

[48] J. C. Roy, F. Salaün, S. Giraud, A. Ferri, G. Chen, and J. Guan, “Solubility of chitin: Solvents, solution behaviors and their related mechanisms,” in *Solubility of Polysaccharides*, InTech, 2017. doi: 10.5772/intechopen.71385.

[49] M. Chandrasekaran, K. D. Kim, and S. C. Chun, “Antibacterial Activity of Chitosan Nanoparticles: A Review,” *Processes*, vol. 8, no. 9, p. 1173, Sep. 2020, doi: 10.3390/pr8091173.

[50] M. Rinaudo, “Chitin and chitosan: Properties and applications,” *Prog. Polym. Sci.*, vol. 31, no. 7, pp. 603–632, Jul. 2006, doi: 10.1016/j.progpolymsci.2006.06.001.

[51] M. Prabaharan, “Review paper: chitosan derivatives as promising materials for controlled drug delivery,” *J. Biomater. Appl.*, vol. 23, no. 1, pp. 5–36, Jul. 2008, doi: 10.1177/0885328208091562.

[52] J. Berger, M. Reist, J. M. Mayer, O. Felt, N. A. Peppas, and R. Gurny, “Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications,” *Eur. J. Pharm. Biopharm.*, vol. 57, no. 1, pp. 19–34, Jan. 2004, doi: 10.1016/s0939-6411(03)00161-9.

[53] T.-L. Yang, “Chitin-based materials in tissue engineering: applications in soft tissue and epithelial organ,” *Int. J. Mol. Sci.*, vol. 12, no. 3, pp. 1936–1963, Mar. 2011, doi: 10.3390/ijms12031936.

[54] H. Yilmaz Atay, “Antibacterial Activity of Chitosan-Based Systems,” in *Functional Chitosan: Drug Delivery and Biomedical Applications*, S. Jana and S. Jana, Eds., Singapore: Springer Singapore, 2019, pp. 457–489. doi: 10.1007/978-981-15-0263-7_15.

[55] P. Gupta, S. Sharma, S. Jabin, and S. Jadoun, “Chitosan nanocomposite for tissue engineering and regenerative medicine: A review,” *Int. J. Biol. Macromol.*, vol. 254, no. Pt 1, p. 127660, Jan. 2024, doi: 10.1016/j.ijbiomac.2023.127660.

[56] M. E. I. Badawy and E. I. Rabea, “Chitosan-Based Edible Membranes for Food Packaging,” in *Bio-based Materials for Food Packaging: Green and Sustainable Advanced Packaging Materials*, S. Ahmed, Ed., Singapore: Springer Singapore, 2018, pp. 237–267. doi: 10.1007/978-981-13-1909-9_11.

[57] L. Wang, Q. Wang, J. Tong, and J. Zhou, “Physicochemical properties of chitosan films incorporated with honeysuckle flower extract for active food packaging,” *J. Food Process Eng.*, vol. 40, no. 1, p. e12305, Feb. 2017, doi: 10.1111/jfpe.12305.

[58] N. E. Suyatma, A. Copinet, L. Tighzert, and V. Coma, “Mechanical and Barrier Properties of Biodegradable Films Made from Chitosan and Poly (Lactic Acid) Blends,” *J. Polym. Environ.*, vol. 12, no. 1, pp. 1–6, Jan. 2004, doi: 10.1023/B:JOOE.0000003121.12800.4e.

[59] Y. A. Shah *et al.*, “Mechanical Properties of Protein-Based Food Packaging Materials,” *Polymers* , vol. 15, no. 7, Mar. 2023, doi: 10.3390/polym15071724.

[60] S. Kumar, A. Mukherjee, and J. Dutta, “Chitosan based nanocomposite films and coatings: Emerging antimicrobial food packaging alternatives,” *Trends Food Sci. Technol.*, vol. 97, pp. 196–209, Mar. 2020, doi: 10.1016/j.tifs.2020.01.002.

[61] “Chitosan market size, share & growth analysis report, 2030.” Dec. 26, 2022. Accessed: Jan. 13, 2024. [Online]. Available: https://www.grandviewresearch.com/industry-analysis/global-chitosan-market

[62] D. Saravanan and T. V. Ayeshvaryaa, “Protein-Based Fibers,” in *Biomedical Applications of Natural Proteins: An Emerging Era in Biomedical Sciences*, D. Kumar and R. R. Kundapur, Eds., New Delhi: Springer India, 2015, pp. 1–30. doi: 10.1007/978-81-322-2491-4_1.

[63] A. Jha, D. K. Mishra, and S. Kumar, “Bombyx mori: Life cycle and as a model organism in life science research,” *J. Entomol. Res.*, vol. 46, no. 2, pp. 378–384, 2022, doi: 10.5958/0974-4576.2022.00068.8.

[64] C. Z. Zhou *et al.*, “Fine organization of Bombyx mori fibroin heavy chain gene,” *Nucleic Acids Res.*, vol. 28, no. 12, pp. 2413–2419, Jun. 2000, doi: 10.1093/nar/28.12.2413.

[65] C. Vepari and D. L. Kaplan, “Silk as a Biomaterial,” *Prog. Polym. Sci.*, vol. 32, no. 8–9, pp. 991–1007, 2007, doi: 10.1016/j.progpolymsci.2007.05.013.

[66] C. Z. Zhou, F. Confalonieri, M. Jacquet, R. Perasso, Z. G. Li, and J. Janin, “Silk fibroin:

structural implications of a remarkable amino acid sequence," *Proteins*, vol. 44, no. 2, pp. 119–122, Aug. 2001, doi: 10.1002/prot.1078.

[67] Y. Takahashi, M. Gehoh, and K. Yuzuriha, "Structure refinement and diffuse streak scattering of silk (Bombyx mori)," *Int. J. Biol. Macromol.*, vol. 24, no. 2–3, pp. 127–138, Mar-Apr 1999, doi: 10.1016/s0141-8130(98)00080-4.

[68] S. Grabska-Zielińska and A. Sionkowska, "How to Improve Physico-Chemical Properties of Silk Fibroin Materials for Biomedical Applications?-Blending and Cross-Linking of Silk Fibroin-A Review," *Materials* , vol. 14, no. 6, Mar. 2021, doi: 10.3390/ma14061510.

[69] S. Rahman, J. Gogoi, S. Dubey, and D. Chowdhury, "Animal derived biopolymers for food packaging applications: A review," *Int. J. Biol. Macromol.*, vol. 255, p. 128197, Jan. 2024, doi: 10.1016/j.ijbiomac.2023.128197.

[70] A. A. Elhadad, A. Alcudia, B. Begines, E. M. Pérez-Soriano, and Y. Torres, "A multidisciplinary perspective on the latest trends in artificial cartilage fabrication to mimic real tissue," *Applied Materials Today*, vol. 29, p. 101603, Dec. 2022, doi: 10.1016/j.apmt.2022.101603.

[71] A. Sagnella *et al.*, "Effect of different fabrication methods on the chemo-physical properties of silk fibroin films and on their interaction with neural cells," *RSC Adv.*, vol. 6, no. 11, pp. 9304–9314, Jan. 2016, doi: 10.1039/C5RA20684G.

[72] H.-Y. Wang, Y.-Q. Zhang, and Z.-G. Wei, "Dissolution and processing of silk fibroin for materials science," *Crit. Rev. Biotechnol.*, vol. 41, no. 3, pp. 406–424, May 2021, doi: 10.1080/07388551.2020.1853030.

[73] D. N. Rockwood, R. C. Preda, T. Yücel, X. Wang, M. L. Lovett, and D. L. Kaplan, "Materials fabrication from Bombyx mori silk fibroin," *Nat. Protoc.*, vol. 6, no. 10, pp. 1612–1631, Sep. 2011, doi: 10.1038/nprot.2011.379.

[74] E. A. Takara, J. Marchese, and N. A. Ochoa, "NaOH treatment of chitosan films: Impact on macromolecular structure and film properties," *Carbohydr. Polym.*, vol. 132, pp. 25–30, Nov. 2015, doi: 10.1016/j.carbpol.2015.05.077.

[75] S.-W. Ha, A. E. Tonelli, and S. M. Hudson, "Structural studies of Bombyx mori silk fibroin during regeneration from solutions and wet fiber spinning," *Biomacromolecules*, vol. 6, no. 3, pp. 1722–1731, May-Jun 2005, doi: 10.1021/bm050010y.

[76] I. Dziedzic and A. Kertmen, "Methods of chitosan identification: History and trends," *Lett. Appl. Nanobioscience*, vol. 12, no. 4, p. 94, Sep. 2022, doi: 10.33263/lianbs124.094.

[77] P. Monti, P. Taddei, G. Freddi, T. Asakura, and M. Tsukada, "Raman spectroscopic characterization of *Bombyx mori* silk fibroin: Raman spectrum of Silk I," *J. Raman*

Spectrosc., vol. 32, no. 2, pp. 103–107, Feb. 2001, doi: 10.1002/jrs.675.

[78] B. Z. Jang, *Advanced Polymer Composites: Principles and Applications*. Taylor & Francis, 1994. [Online]. Available: https://play.google.com/store/books/details?id=OYp5QgAACAAJ

[79] G. C. Jacob, J. M. Starbuck, J. F. Fellers, S. Simunovic, and R. G. Boeman, "Strain rate effects on the mechanical properties of polymer composite materials," *J. Appl. Polym. Sci.*, vol. 94, no. 1, pp. 296–301, Sep. 2004, doi: 10.1002/app.20901.

[80] J. Xu, K. Liu, W. Chang, B.-S. Chiou, M. Chen, and F. Liu, "Regulating the Physicochemical Properties of Chitosan Films through Concentration and Neutralization," *Foods*, vol. 11, no. 11, Jun. 2022, doi: 10.3390/foods11111657.

[81] "CWF® 1000 clear polystyrene," Multi-Plastics. Accessed: Mar. 31, 2024. [Online]. Available: https://multi-plastics.com/products/cwf-1000-clear-polystyrene/

[82] "LDPE," Curbell Plastics. Accessed: Apr. 01, 2024. [Online]. Available: https://www.curbellplastics.com/materials/plastics/ldpe/

[83] "Polyester Film," Curbell Plastics. Accessed: Apr. 01, 2024. [Online]. Available: https://www.curbellplastics.com/materials/plastics/polyester-film/

[84] N. Wrońska, N. Katir, M. Nowak-Lange, A. El Kadib, and K. Lisowska, "Biodegradable Chitosan-Based Films as an Alternative to Plastic Packaging," *Foods*, vol. 12, no. 18, Sep. 2023, doi: 10.3390/foods12183519.

[85] A. T. Zvinavashe *et al.*, "Degradation of Regenerated Silk Fibroin in Soil and Marine Environments," *ACS Sustainable Chem. Eng.*, vol. 10, no. 34, pp. 11088–11097, Aug. 2022, doi: 10.1021/acssuschemeng.2c00949.

[86] G. H. Altman and B. D. Farrell, "Sericulture as a sustainable agroindustry," *Cleaner and Circular Bioeconomy*, vol. 2, p. 100011, Aug. 2022, doi: 10.1016/j.clcb.2022.100011.

[87] W. Chen, "Analysis on Operation of Chinese Cocoon Silk Industry in 2020 and Prospect in 2021," *Journal of Silk*, vol. 58, Dec. 2021, [Online]. Available: https://inserco.org/en/sites/default/files/journal_of_silk_2021_special_Issue.pdf

[88] W. DeWit, E. T. Burns, J. C. Guinchard, and N. Ahmed, "Plastics: the costs to society, the environment and the economy," *World Wide Fund for Nature: Gland*.

[89] M. F. Astudillo, G. Thalwitz, and F. Vollrath, "11 - Life cycle assessment of silk production – a case study from India," in *Handbook of Life Cycle Assessment (LCA) of Textiles and Clothing*, S. S. Muthu, Ed., Woodhead Publishing, 2015, pp. 255–274. doi: 10.1016/B978-0-08-100169-1.00011-3.

[90] V. Mannheim, “Life Cycle Assessment Model of Plastic Products: Comparing Environmental Impacts for Different Scenarios in the Production Stage,” *Polymers* , vol. 13, no. 5, Mar. 2021, doi: 10.3390/polym13050777.

[91] “Lithium bromide.” Accessed: Mar. 13, 2024. [Online]. Available: https://www.sigmaaldrich.com/US/en/product/sigald/213225

[92] “Casein technical grade 9000-71-9.” Accessed: Mar. 14, 2024. [Online]. Available: https://www.sigmaaldrich.com/US/en/product/sigma/c7078?utm_source=google&utm_medium=cpc&utm_campaign=8906396340&utm_content=89360792146&gclid=Cj0KCQjwwMqvBhCtARIsAIXsZpYAyvezHVSs0EEdR3L7JQ98qdMI92NfM2pAYnLhAeDX3_3XYAH68SwaAitVEALw_wcB

[93] L. Chen, Z. Yang, D. J. McClements, Z. Jin, and M. Miao, “Chapter 20 - Biological macromolecules for nutrients delivery,” in *Biological Macromolecules*, A. K. Nayak, A. K. Dhara, and D. Pal, Eds., Academic Press, 2022, pp. 455–477. doi: 10.1016/B978-0-323-85759-8.00020-8.

[94] Zion Market Research, “Casein and caseinate market by casein type (rennet casein and acid casein), by caseinate type (calcium, sodium, potassium, and others), by application (food & beverages, pharmaceuticals, and dietary supplements), and by region - global and regional industry overview, market intelligence, comprehensive analysis, historical data, and forecasts 2023 – 2030.” Mar. 06, 2024. Accessed: Mar. 16, 2024. [Online]. Available: https://www.zionmarketresearch.com/report/casein-and-caseinate-market

[95] M. D. Shoulders and R. T. Raines, “Collagen structure and stability,” *Annu. Rev. Biochem.*, vol. 78, pp. 929–958, 2009, doi: 10.1146/annurev.biochem.77.032207.120833.

[96] “Collagen Market Size, Share, Growth & Trends Report, 2030.” Jan. 22, 2024. Accessed: Mar. 17, 2024. [Online]. Available: https://www.grandviewresearch.com/industry-analysis/collagen-market

[97] L. D. Campos, V. de A. Santos Junior, J. D. Pimentel, G. L. F. Carregã, and C. B. B. Cazarin, “Collagen supplementation in skin and orthopedic diseases: A review of the literature,” *Heliyon*, vol. 9, no. 4, p. e14961, Apr. 2023, doi: 10.1016/j.heliyon.2023.e14961.

[98] B. Wang, W. Yang, J. McKittrick, and M. A. Meyers, “Keratin: Structure, mechanical properties, occurrence in biological organisms, and efforts at bioinspiration,” *Prog. Mater Sci.*, vol. 76, pp. 229–318, Mar. 2016, doi: 10.1016/j.pmatsci.2015.06.001.

[99] F. Costa, R. Silva, and A. R. Boccaccini, “7 - Fibrous protein-based biomaterials (silk, keratin, elastin, and resilin proteins) for tissue regeneration and repair,” in *Peptides and Proteins as Biomaterials for Tissue Regeneration and Repair*, M. A. Barbosa and M. C.

L. Martins, Eds., Woodhead Publishing, 2018, pp. 175–204. doi: 10.1016/B978-0-08-100803-4.00007-3.

[100] "Keratin Products Market Size, Share, Competitive Landscape and Trend Analysis Report by Product Type, by Application, by Form : Global Opportunity Analysis and Industry Forecast, 2023-2032," Allied Market Research. Accessed: Mar. 17, 2024. [Online]. Available: https://www.alliedmarketresearch.com/keratin-products-market-A12406

[101] P.-L. Pan *et al.*, "A comprehensive omics analysis and functional survey of cuticular proteins in the brown planthopper," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 115, no. 20, pp. 5175–5180, May 2018, doi: 10.1073/pnas.1716951115.

[102] M. Burrows, S. R. Shaw, and G. P. Sutton, "Resilin and chitinous cuticle form a composite structure for energy storage in jumping by froghopper insects," *BMC Biol.*, vol. 6, p. 41, Sep. 2008, doi: 10.1186/1741-7007-6-41.

[103] F. Vollrath, R. Carter, G. Rajesh, G. Thalwitz, and M. F. Astudillo, "Life cycle analysis of cumulative energy demand on sericulture in Karnataka, India," 2013, [Online]. Available: https://www.academia.edu/download/32243270/Silk_LCA_Vollrath_et_al_2013.pdf

[104] G. Cheng *et al.*, "Insignificant Difference in Biocompatibility of Regenerated Silk Fibroin Prepared with Ternary Reagent Compared with Regenerated Silk Fibroin Prepared with Lithium Bromide," *Polymers* , vol. 14, no. 18, Sep. 2022, doi: 10.3390/polym14183903.

[105] D. M. de C. Bittencourt, P. Oliveira, V. A. Michalczechen-Lacerda, G. M. S. Rosinha, J. A. Jones, and E. L. Rech, "Bioengineering of spider silks for the production of biomedical materials," *Front Bioeng Biotechnol*, vol. 10, p. 958486, Aug. 2022, doi: 10.3389/fbioe.2022.958486.

[106] K. Kurihara, R. Hokari, and N. Takada, "Capillary Effect Enhancement in a Plastic Capillary Tube by Nanostructured Surface," *Polymers* , vol. 13, no. 4, Feb. 2021, doi: 10.3390/polym13040628.

[107] A. F. Stalder, G. Kulik, D. Sage, L. Barbieri, and P. Hoffmann, "A snake-based approach to accurate determination of both contact points and contact angles," *Colloids Surf. A Physicochem. Eng. Asp.*, vol. 286, no. 1–3, pp. 92–103, Sep. 2006, doi: 10.1016/j.colsurfa.2006.03.008.

[108] Y. Liu *et al.*, "Preparation and characterization of chitosan films with three kinds of molecular weight for food packaging," *Int. J. Biol. Macromol.*, vol. 155, pp. 249–259, Jul. 2020, doi: 10.1016/j.ijbiomac.2020.03.217.

[109] D. R. Smith, A. P. Escobar, M. N. Andris, B. M. Boardman, and G. M. Peters, "Understanding the Molecular-Level Interactions of Glucosamine-Glycerol Assemblies: A Model System for Chitosan Plasticization," *ACS Omega*, vol. 6, no. 39, pp. 25227–25234, Oct. 2021, doi: 10.1021/acsomega.1c03016.

[110] S. Lu *et al.*, "Insoluble and flexible silk films containing glycerol," *Biomacromolecules*, vol. 11, no. 1, pp. 143–150, Jan. 2010, doi: 10.1021/bm900993n.

[111] K. Li *et al.*, "Glycerin/NaOH Aqueous Solution as a Green Solvent System for Dissolution of Cellulose," *Polymers* , vol. 12, no. 8, Aug. 2020, doi: 10.3390/polym12081735.

[112] J. Van Gerpen and G. Knothe, "16 - Bioenergy and Biofuels from Soybeans," in *Soybeans*, L. A. Johnson, P. J. White, and R. Galloway, Eds., AOCS Press, 2008, pp. 499–538. doi: 10.1016/B978-1-893997-64-6.50019-6.

[113] W. Janik *et al.*, "Chitosan-based films with alternative eco-friendly plasticizers: Preparation, physicochemical properties and stability," *Carbohydr. Polym.*, vol. 301, no. Pt A, p. 120277, Feb. 2023, doi: 10.1016/j.carbpol.2022.120277.

[114] D. Klocke and H. Schmitz, "Water as a major modulator of the mechanical properties of insect cuticle," *Acta Biomater.*, vol. 7, no. 7, pp. 2935–2942, Jul. 2011, doi: 10.1016/j.actbio.2011.04.004.

[115] K.-Y. Law, "Definitions for Hydrophilicity, Hydrophobicity, and Superhydrophobicity: Getting the Basics Right," *J. Phys. Chem. Lett.*, vol. 5, no. 4, pp. 686–688, Feb. 2014, doi: 10.1021/jz402762h.

[116] I. S. Bayer, "Superhydrophobic coatings from ecofriendly materials and processes: A review," *Adv. Mater. Interfaces*, vol. 7, no. 13, p. 2000095, Jul. 2020, doi: 10.1002/admi.202000095.