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Silk Based Bioinks for Soft Tissue Reconstruction Using 3-Dimensional (3D) Printing with *in vitro* and *in vivo* Assessments

María J. Rodriguez¹, Joseph Brown¹, Jodie Giordano¹, Samuel J. Lin², Fiorenzo G. Omenetto¹, and David L. Kaplan¹

¹Department of Biomedical Engineering, Tufts University, Medford, MA, USA

²Division of Plastic and Reconstructive Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, 02215

Abstract

In the field of soft tissue reconstruction, custom implants could address the need for materials that can fill complex geometries. Our aim was to develop a material system with optimal rheology for material extrusion, that can be processed in physiological and non-toxic conditions and provide structural support for soft tissue reconstruction. To meet this need we developed silk based bioinks using gelatin as a bulking agent and glycerol as a non-toxic additive to induce physical crosslinking. We developed these inks optimizing printing efficacy and resolution for patient-specific geometries that can be used for soft tissue reconstruction. We demonstrated *in vitro* that the material was stable under physiological conditions and could be tuned to match soft tissue mechanical properties. We demonstrated *in vivo* that the material was biocompatible and could be tuned to maintain shape and volume up to three months while promoting cellular infiltration and tissue integration.

1. Introduction

Three-dimensional printing (3DP) of physiologically relevant tissue reconstructions have already proved to be an invaluable tool for surgical planning and prosthetics [1]. For medical prostheses in the field of tissue engineering, 3DP technology supports the custom design of complex multi-material implants for patient-specific geometries [2]. A major remaining challenge in the field is in materials (i.e. ink) development [3]. Printable materials should have rheological properties to permit extrusion and solidification upon deposition into mechanically robust printed 3D structures. For medical implants, the materials must be biocompatible and fully degradable while promoting tissue regeneration and proper functionality throughout the lifetime of the device. Additionally, pre- or postprocessing of the material must maintain biocompatibility in order to support incorporation of biological components (e.g., cells, cytokines, and growth hormones) into printed structures. Several recent advancements have utilized biocompatible polymeric materials such as alginate [4],

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gelatin [5], fibrin [6], and collagen [7]. Often these materials generate relatively weak structures, which degrade or remodel too rapidly *in vivo*, thus imposing the use of crosslinking methods to improve volume retention, strengthen mechanics and maintain structural features. Unfortunately, these additives are problematic due to the added complexity and issues of poor homogeneity in material property outcomes, or due to inherent cytotoxicity of residual compounds after crosslinking.

To address this issue, silk protein-based bioinks have recently been studied with numerous advantageous features, including: all aqueous and ambient processing conditions, cytocompatible crosslinking methods, robust mechanical features and full degradability to amino acids [8], [9]. Silk biomaterials have received FDA approval for some medical products, including sutures and surgical meshes, and can be processed into a variety of material formats [10]–[12].

Finding a suitable soft tissue replacement material can address a wide range of clinical needs expanding from cosmetic, reconstructive and correctional indications. With 5 million reconstructive surgeries [13] being performed annually there is a growing need for alternative methods and materials which can enhance soft tissue regeneration. Historically soft tissue augmentation has relied on tissue transfer of autologous tissue such as adipose tissue [14]. Despite advances in surgical techniques autologous free fat transfer is remains inconsistent with variable success reported [15] The main challenges with these techniques are low long term reliability due to variable reabsorption rates from 20 to 90% of their volume and lack of vascularization leading to donor site morbidity over time [15]–[18]. Because of these disadvantages, numerous natural, synthetic, and hybrid materials have been used to act as adipose surrogates [19], [20]. The ability to specifically tailor a synthetic polymer with respect to its mechanical properties, chemical properties, and degradability are some advantages of using synthetic fillers; however these materials can exhibit challenges such as foreign body reaction, comparatively high infection rates, need for re-operation, and cost. Naturally occurring materials have the advantage of being largely biocompatible and able to match native tissue mechanical and biological properties [20]. A useful soft tissue replacement material would enhance volume retention, be biocompatible, and match the mechanical properties of native adipose tissue. Tissue printing technology offers a unique prospect to produce biologic substitutes that promote tissue regeneration and restore function by printing growth factors or other biological materials along with matrix materials in a defined and organized manner [21]. We have previously shown that silk biomaterials can support the formation and regeneration of soft tissue both *in vitro* and *in vivo* while maintaining volume retention over 1 year *in vivo* [22]–[27]. Our past fabrication methods, based on molding and freeze drying, limited implant designs to more standard shapes. 3-Dimensional Printing allows for the custom generation of patient-specific geometries. Silk-gelatin blends have been used for material extrusion 3-D printing [28]. However, these studies used a crosslinking mechanism which resulted in poor printing resolution. While appropriate for smaller, cell-laden structures, it would be expected that these mechanisms would not be appropriate for larger constructs which would need to be stable while printing for longer periods of time.

The aim of the current study was to evaluate a range of silk-based ink formulations for extrusion based printing efficacy, resolution, mechanical rigidity and biocompatibility, with utility for soft tissue reconstruction. Extrusion based printing requires material formulations to be optimized due to need for a controllable, fast phase transition to allow for 3-dimensional structures to be fabricated. Previous work in the extrusion of silk focused on developing a set of biocompatible inks (i.e. bioinks) that were physically stabilized through the addition of polyols [8]. In the current work gelatin was incorporated as a bulking agent to create patient specific implants for soft tissue reconstruction. By using silk as the structural matrix, materials were generated where degradation and mechanical properties can be tuned. The shear thinning properties of silk make it useful for material extrusion and in combination with glycerol generates insoluble structures without the need for toxic crosslinking methods. Gelatin undergoes a temperature controlled phase transition under physiological conditions making it useful as a bulking agent to create three-dimensional structures under physiological conditions. Gelatin is biocompatible and has been widely used in 3D printing [29]–[34]. However the mechanical properties of gelatin limit its applications. We hypothesized that blending these three components (silk, gelatin, and glycerol) could lead to a biomaterial composite formulation ideal for 3DP of soft tissue.

2. Methods

2.1. Bioink Fabrication

Aqueous silk fibroin solution was prepared according to previously published methods [10]. Briefly, silkworm *Bombyx mori* silkworm cocoons, purchased from Tajima Shoji Co. (Yokohama, Japan), were extracted in 0.02 M sodium carbonate to remove the sericin coating from the fibers. Fibers were then rinsed in distilled water, dissolved in 9.3 M Lithium Bromide, and dialyzed against distilled water to remove the salt. The resulting 6% w/v silk solution was concentrated to 20% w/w and stored at 4°C until use. Glycerol and gelatin were purchased from Sigma Aldrich (St. Louis, MO, US). Glycerol was diluted to a 700 mg/ml in distilled water and gelatin was solubilized to a 400 mg/ml working solution and stored at 4°C until use.

Based on previous work [35] glycerol was mixed with the silk solution at a fixed ratio of (3:1) weight: weight (w/w) silk to glycerol. The aqueous gelatin solution (with different concentrations depending on the sample, see Table 1 and 2, was mixed into the silk-glycerol solution, vortexed for 1 minute and stored at 60°C for 10 minutes. To homogenize solutions this procedure was repeated three times. The solution was then stored at 60°C until removal of all air bubbles. The resulting bioink was used within 24 hours of mixing and stored at 4°C until used.

2.2. Stability

In order to ensure the stability of printed materials in aqueous solution, increasing silk-glycerol to gelatin (w/w) ratios were studied including five different conditions with four samples per condition (Table 1). The different bioinks were poured onto petri dishes and solidified at 4°C for 1 hour. The resulting gels were physically crosslinked by immersion in 500 mg/ml glycerol solution for 1 hour. The gels were placed on a rotary shaker and then

moved to phosphate buffered saline (PBS) solution and incubated at 37°C for mass loss studies. All samples were weighed prior to immersion into the glycerol bath. At 1 hour after glycerol immersion, samples were removed, rinsed with deionized water, dabbed dry and weighed. The samples were then moved to PBS and incubated for the remaining time points (3 hours, 5 hours, 8 hours, 1 day, 3 days and 2 weeks). At each time point the above procedure was repeated. The weights of each time point were recorded, normalized to the 1 hour after glycerol weights and the data are reported as percentage change as in equation (1):

$$\text{Mass remaining (\%)} = \frac{M_2 - M_1}{M_1} \times 100$$

Where M_2 is the mass at each individual time point and M_1 is the initial mass after 1 hour glycerol bath.

2.3. Secondary Structure Analysis

Protein secondary structures were determined through Fourier Transfer Infrared Spectroscopy (FTIR, using a JASCO FTIR 6200 spectrometer (JASCO, Tokyo, Japan) combined with a MIRacle™ attenuated total reflection (ATR) germanium crystal. Background and spectral scans were measured from 4000–600 cm^{-1} at a resolution of 2 cm^{-1} for 32 scans per sample. Three different post processing conditions were evaluated, no post processing, immersion in methanol (90% w/v) and immersion in glycerol (50% v/v). Silk only, silk-glycerol, gelatin only and gelatin-glycerol were used as controls. Bioink formulations of silk gelatin and silk gelatin glycerol were compared against controls. To preserve protein secondary structures samples were air dried for 24 hours in a desiccator.

2.4. Bio Printer and Interfacing Software

As previously published [8] a material extrusion bio printer built in-house was used for 3DP. This system is composed of by five 2-phase unipolar 1.8° stepper motors which drive linear motion via translation lead screws with rolled 1–4"-20 threads. Three stepper motors were used for X, Y and Z motion control. These generate maximum 6.35 μm of linear movement per discrete rotary motor step and minimum of 0.8 μm when 1/8 micro stepping is used. Two stepper motors were used to drive two syringe plungers for extrusion. The linear movement of these motors for a 10mLBD Luer-Lok™ syringe with a plunger cross sectional area of 324 mm^2 results in 128.628 nL per discrete rotary motor step. The bio printer is controlled by Arduino Mega and a motor shield Ultimaker 1.7 which can interface with desktop slicing and printing algorithms. These controllers enable X, Y, Z motion control and two automated heaters. Automated temperature control was modulated via 12 V 40W ceramic cartridge heaters to temperature and 1.25 mm × 2.0 mm 100 K NTC thermistors (temperature range: –40 to 270°C) were used for temperature measurement. Repetier Host open source software was used for interface between Computer-aided drafting (CAD) files created and the slicing software (Slic3r) which automatically produced print programming.

2.5. Extrusion Force Measurement

The extrusion force expected during printing was evaluated to understand the conditions that are expected during printing and to ensure that the solution behavior of the bioinks were stable under these conditions. The Slicr3 slicing software [36] calculates flow rate (material to be extruded per distance unit) based on layer height and extrusion width. Slicr3 uses a flow math algorithm to calculate the flow rate needed according the several parameters such as perimeters, first layer settings, and nozzle diameter [36]. This is an estimation which needs to be calibrated for each material used and optimized according to the print geometry. Therefore, an accurate flow rate would be dependent on the bio ink used. In order to create an empirical model not dependent on flow rate the forces experienced during printing were considered. A custom experimental set up was used (Supplementary Figure 1). A 25 N flexi force sensor (A201 Tekscan, Boston, MA) was placed in direct contact with the syringe plunger. A LabVIEW™ (National Instruments) program was created to collect the analog voltage data. The flexi-force sensor was first calibrated using manufacturer standards. The calibration resulted in a standard curve relating the measured voltage to force. This calibration curve was used to convert the incoming voltage to force in the LabVIEW program. The force experienced during printing was measured first for water as calibration then for the silk-gelatin bio ink. These values were then used as input conditions for a Finite Element Model (FEM). This model described in detail in the following section was used to assess stresses for a range of extrusion forces, materials, and nozzle geometries. The results from this model were the shear rates and stresses that would be expected while printing.

2.6. Finite Element Simulation of Flow in Syringe

Laminar flow through a syringe was simulated using Comsol 4.0a (Comsol, Palo Alto, CA). A 10 mL BD syringe was used in the simulation with an inner diameter of 16.2 mm and a length of 95.2 mm. A typical needle size range used in printing was 21 to 25 gauge which correspond to inner diameters from 300 μm to 600 μm . A 500 μm needle was used for initial simulations and parametric studies were done to derive the range of stresses produced with varying needle size. The geometry was meshed using free tetrahedral elements. A laminar flow was evaluated using boundary conditions of normal stress at the inlet and atmospheric pressure at the outlet. The normal stress was derived from experimental test described above. An incompressible non-Newtonian fluid model was used to simulate flow of bioinks using a shear rate dependent relationship and an extrusion temperature of 37°C. An incompressible fluid of silk-glycerol-gelatin was assumed to have a constant density of 1 kg/m^3 , and empirically derived constants m and n of 1.14 and 0.97 respectively. The procedure describing how these parameters were derived is detailed in the Fluid Flow section below. The purpose of the simulation was to evaluate the shear rates that would be experienced during printing at deposition temperatures in order to more accurately characterize the rheology of bioinks.

2.7. Dynamic Viscoelastic Behavior

Rheological measurements were performed to characterize dynamic viscoelastic behavior of the bioinks. Gelation is controlled by the thermoreversible behavior of gelatin. This thermoreversible behavior was characterized by evaluating the viscoelastic properties of

bioinks while heating over a range of temperatures. All dynamic rheology studies were carried out on TA Instruments ARES-LS2 rheometer (TA Instruments, New castle, DE) using 50mm stainless steel conical plate (angle: 0.0402 rad) and a temperature controlled Peltier plate. Samples were prepared as specified in bio ink formulation section and were stored at 60°C until tested. Prior to testing the samples were mixed by gentle pipetting and 420 µL was loaded onto rheometer plate. Samples were loaded at 37°C and were fluid at this point. The cone was lowered to specified gap and low viscosity mineral oil was placed on the edges to prevent evaporation of water.

A strain sweep test revealed that 1% strain at 1 Hz frequency was within the linear viscoelastic region (LVR) for these formulation. The dynamic temperature sweep was performed at a constant strain of 1% and a frequency of 1 Hz and was carried out in three steps following literature standards[37]:

- a. Cooling: Temperature was lowered to 10°C at a cooling rate of 2°C/min.
- b. Equilibration: Samples were equilibrated for 15 minutes at 10°C.
- c. Heating: a temperature sweep from 10°C to 40°C was carried out at a heating rate of 2°C/min.

The melting temperature was calculated as the temperature where there was an appreciable decrease in complex viscosity (η^*). The complex viscosity was defined as in equation (2):

$$\eta^* = \sqrt{\frac{G'^2 + G''^2}{\omega}}$$

where G' is the storage modulus, G'' is the loss modulus and ω is the frequency. The appreciable change in complex viscosity was determined as the crossover temperature where the storage modulus equals the loss modulus which is the ratio of G' to G'' , equals to 1.

2.8. Flow Behavior

The flow properties of the silk-gelatin bioinks were measured under steady shear at 37°C on TA Instruments ARES-LS2 rheometer (TA Instruments, New castle, DE) using 50mm stainless steel conical plate (angle: 0.0402 rad) and a temperature controlled Peltier plate. The TA Instruments Orchestrator V7.2.0.4 software was used for curve fitting analysis. The samples were prepared as described above stored at 60°C until tested, loaded at 37°C and equilibrated for 15 minutes prior to testing. A steady shear rate ($\dot{\gamma}$) ramp was applied from 1 – 1000 s⁻¹, 10 points were taken per decade and the resulting shear stress (τ) was measured. The resulting steady shear viscosity (η) was calculated as shear stress over shear rate ($\frac{d\tau}{d\dot{\gamma}}$) as in equation (3):

$$\eta = \frac{\tau}{\dot{\gamma}}$$

A flow curve was obtained by plotting the log of viscosity against the log of shear rates applied. The data was fitted to different rheological models using the TA Orchestrator

software and the best fit model was selected on the basis of standard error. The Power Law Model was used to describe non-Newtonian fluids that exhibit shear-thinning with a constant slope [38] described in equation (4):

$$\eta = m(\dot{\gamma})^{n-1}$$

In the above equation η and $\dot{\gamma}$ were parameters derived from flow curves and m and n are curve fitting coefficients calculated to obtain best fit to data.

2.9. 3-Dimensional Printing

Bio ink formulations were determined to be optimal based on: a) flow behavior that would allow for stable extrusion in the range of shear rates expected during printing, b) physiologically relevant melting (30 to 40°C) and deposition temperatures (20–25°C), and c) formation of insoluble material when incubated under physiological conditions. The optimal formulations were printed using the custom built 3D printer. The bioink was placed in a heated syringe cartridge and equilibrated to its optimal printing temperature. After printing the constructs were left to dry for 15 minutes and were placed in a glycerol bath for 1 hour. All geometries to be printed were designed in Solidworks (Dassault Systèmes, Waltham, MA, USA) and converted into an (Stereo lithography) STL file. As described above these files were then imported into Slic3r to generate G-code instructions for the 3D printer and sent to the printer via Repertier Host. The print line definition of bio ink blends was evaluated microscopically and with profilometry. Lines 10 mm in length, with 1, 3, 5 and 10 layers were printed onto glass slides. Three lines were printed per layer condition. A Veeco Dektak 6M stylus profilometer (Bruker Corp, Billerica, MA) was used to measure the topography and profile of the printed lines. The prints were deposited on a glass slide that was first coated with a thin layer of gold using standard sputtering techniques (SC7620 “Mini” Sputter Coater/Glow Discharge System, Quorum Technologies, East Sussex, and England). A scan length of 5 mm and a scan range of 65 to 200 μm were used to scan across printed lines at three separate locations. The average sample height was calculated as the difference in height of the glass slide to the top of the sample. The 3-D print resolution was analyzed with phase contrast microscopy (Keyence, BZ-X700 series, Itasca, IL).

2.10. Mechanical Testing

Unconfined compression testing was conducted to estimate the mechanical properties of the material. Bioinks were formed in a petri dish and left to gel at room temperature for 1 hour. The gels were then moved onto a glycerol bath and crosslinked as described previously. The crosslinked gels were biopsy punched into cylinders (8 mm diameter and 3 mm height). Each sample diameter was recorded for calculations of stress. The samples were incubated in PBS for 12 hours before testing. Samples were loaded in a TA Instruments RSA3 Dynamic Mechanical Analyzer (TA Instruments, New Castle, DE) between stainless steel parallel plates in an immersion PBS bath. A preload of 2 g was used to ensure contact with the sample. Samples were subjected to five preloading load and unload cycles in order to eliminate artifacts. Samples were strained from 0 to 20% strain during this preload phase and the sixth cycle was recorded. The resulting stress data was plotted against strain. Using a

linear regression fit offset to the linear portion of the curve the elastic moduli was calculated at 5–10% strain. All testing was conducted at a constant crosshead speed of 1 mm min⁻¹. Samples were allowed to recover for 30 minutes. A strain sweep at a constant frequency of 1 Hz was used to establish the linear viscoelastic region (LVR). A frequency sweep was performed at 1% strain which was within the LVR. The recovery was calculated by comparing the height of the sponge (with applied 0.5 gram preload) pre vs. post 20% axial strain. Hysteresis was measured as the ratio of the area under the curve between the loading and unloading regions of each strain sweep. The compression modulus was calculated as the slope of the linear elastic region at 1–5% and 5–10% axial strains.

2.11. 3-Dimensional (3D) Printing of Cheek Implants

To demonstrate applicability in patient specific implants, Digital Imaging and Communications in Medicine (DICOM) images were extracted from CT-scans of a patient with a head and neck tumor mass. These CT scans were archived studies obtained in standard fashion for clinical evaluation and provided by collaborators in Beth Israel Deaconess Medical Center. This study was approved by the Beth Israel Deaconess Institutional Review Board. These DICOM images were segmented using open source image software 3D Slicer [39]. The patient had a tumor removed and his soft tissue was replaced by vascularized autologous flaps. In this case an alternate material whose properties matched that of the soft tissue could be used. The cheek geometry was extracted from DICOM images using a threshold segmentation and a 3D model was reconstructed. This was exported as an STL mesh and was modified to a printable format using open source software Mesh Lab.

2.12. *In-vivo* Implantation Biocompatibility

All *in vivo* experimentation was conducted under protocols approved by the Tufts Institutional Animal Care and Use Committee (M2014-02 P-13). Mice used in this study were 4–6 week old BALBc female mice (Charles River Laboratories, Wilmington, MA). The mice were randomly assigned into three time points with three animals per time point. Each animal had one 3D printed construct and one 3D printed lyophilized sample. Bio ink formulations were 10% w/v silk, 25% w/w glycerol, 10% w/v gelatin and were prepared using standard aseptic technique [40]. Silk solution was sterile filtered and gelatin solution was autoclaved for sterility. Cylinders of 4 mm diameter 2 mm thickness were printed in a custom built 3D printer [41] inside a laminar flow hood and were stored sterile at 4°C until implantation. The samples were subcutaneously implanted in lateral pockets under general anesthesia of oxygen and isoflurane. At week 2, month 1, and month 3 post-implantation, animals were euthanized and the specimens along with the adjacent tissues were collected for histological examination.

2.13. Histochemistry

Explants were fixed in 10% neutral buffered formalin (NBF) and embedded in paraffin following xylene and graded ethanol incubation. Samples were sectioned to 6 µm thickness, placed on glass slides and paraffin was removed. Sections were then stained with hematoxylin and eosin (H&E) (Sigma-Aldrich, St. Louis, MO) to visualize cell nuclei and

cytoplasm. Following staining samples were imaged with 4× and 20× objectives using an inverted microscope (Keyence, BZ-X700 series, Itasca, IL).

2.14. Statistical Analysis

Data are expressed as mean $\pm$ standard deviation. One or two-way analysis of variance (ANOVA) and Tukey post-hoc analysis were used to determine statistically significant differences. Statistical significance was accepted at the $p < 0.05$ level and indicated in figures and tables as *p.

3. Results

The printability of the bioinks was qualitatively assessed to establish the minimum and maximum limits of gelatin that could be printed. A minimum of 5% w/v of gelatin was needed to retain a solid ink structure upon deposition. Gelatin concentrations above 15% w/v resulted in frequent clogging of the syringe needle tip. These qualitative assessments were useful in narrowing the 3D-printable formulations to consider further in the study. To relate these observations to quantitative characteristics of the bioinks, the stability of the printed material and the rheological properties of the varying silk-glycerol-gelatin formulations were assessed.

To establish the silk to gelatin ratio that would be stable in both aqueous environment and physiological temperatures, the ratios were evaluated as shown in Table 1. Additionally sample conditions with no post processing were tested (data not shown) but solubilized completely within one hour of incubation and therefore were not included in the remaining time points.

The percentage of mass remaining after incubation for two weeks at 37°C is shown in Figure 1. (A) Mass of printed gel remaining with increasing silk to gelatin ratio after incubation up to 5 weeks. Silk : Gelatin : Glycerol in ratios of 2:1, 1:1, 1:2 were tested with two different post processing conditions glycerol bath immersion and methanol bath immersion. (B) Images of phase separation induced by varying silk to gelatin ratios, scale bars represent 1 mm. Four samples per condition were tested and the average mass remaining is shown with error bars indicating the standard deviation of the replicates.

Overall comparable behavior was seen for both treatment conditions with the 2:1 samples having the highest mass fraction remaining and the 1:2 samples being unstable and solubilizing within 2 weeks for glycerol bath immersion and 3 weeks for methanol bath immersion. For both glycerol and methanol treatment a 1:1 ratio of silk to gelatin the material stability and homogeneity was sufficient for the goals of the study. The higher gelatin to silk ratios led to phase separation upon the physical crosslinking of the silk in glycerol, and this led to unstable structures when incubated at 37°C. Supplementary Figure 2 shows this change in morphology over a four week period.

FTIR analysis of the amide I region ($1600 - 1700 \text{ cm}^{-1}$) can provide insight into protein secondary structure specific to intermolecular protein order and bonding in comparison to disordered amorphous structures. The physical crosslinking of formulations was tested to

analyze the effects of post processing treatments on protein secondary structure. We hypothesized that the insolubility and stability seen in the mass loss study for the samples immersed in a glycerol bath would result in a beta sheet structure comparable to that of methanol treatment. Silk: glycerol: gelatin with a 1:1 ratio with no post processing, post methanol immersion and post glycerol immersion were evaluated. Controls consisted of silk and silk glycerol samples.

The self-assembly process for the silk fibroin protein that produces an insoluble material is accompanied by an increase in the β -sheet structure or silk II crystalline structures and a reduction of silk I or random coil structure [35], [42], [43]. We hypothesized that the insolubility induced by post printing glycerol immersion was due to the formation of beta sheet secondary structure in the silk protein. FTIR analysis of the amide I region ($1600 - 1700 \text{ cm}^{-1}$) provides insight into protein secondary structure specific to intermolecular protein order and bonding in comparison to disordered amorphous structures. For silk fibroin, β -sheet structure is typically observed with peaks between $1616-1637 \text{ cm}^{-1}$, while amorphous elements such as random coils and α -helices occur with peaks between $1638-1662 \text{ cm}^{-1}$ [43]. The resulting FTIR spectra of the silk: gelatin: glycerol samples showed appearance of the characteristic beta sheet peaks at 1624 cm^{-1} (indicated by dashed line in Figure 1D) and the reduction of the random coil peak at 1648 cm^{-1} for both methanol treated (Figure 1D(f)), glycerol treated (Figure 1D(e)), and no post treatment (Figure 1D(d)) samples. These spectra are similar to the silk only controls with no post treatment (Figure 1D(a)) where only the amorphous silk I peak was present and the methanol treated (Figure 1D(c)), and glycerol treated (Figure 1D(b)) where the amorphous peak was reduced.

The bioinks were tested under steady shear at 37°C to characterize fluid behavior (Supplemental Figure 1). This behavior was tested for a 1:1 ratio 10% Silk: 10% Gelatin w/v. The power law model was fitted to the average flow curve (Supplementary Figure 1). All samples yielded a confidence fit of 0.95.

We anticipated that higher percentages of gelatin would lead to faster gelation (physical crosslinking and beta sheet formation) of the ink and more stable structures. To quantitatively test this the rheological properties of the bioinks was assessed with varying silk-gelatin concentrations (Supplementary Table 2). The resulting temperature sweep curves for the conditions tested along with the calculated melting temperatures are shown in Figure 2.

The transition from fluid like behavior ($G'' > G'$) to solid like behavior ($G' > G''$) indicates the melting temperature of the polymer blend. There was no statistical difference in the melting temperatures of bio ink formulations with increasing silk concentration, while there was a statistically significant increase in melting temperature with increasing concentration of gelatin. This indicates that the temperature controlled gelation of the bioinks was driven by gelatin. The data also showed that above the melting temperature the polymer blend has a stable Newtonian fluid behavior and below this temperature a stable solid behavior. This is very useful for printing applications by keeping the extrusion temperature above the T_m and the deposition temperature below the T_m . These results also indicate that to avoid having to cool the deposition surface at least 10% w/v of gelatin is needed. In order to better

understand the fluid flow during extrusion the laminar flow was simulated through a syringe and needle using Comsol. The forces experienced during a normal printing cycle were measured (Supplemental Figure 1). During a standard cycle the forces remained fairly consistent during printing and ranged from 1 to 2 Newton. The shear stresses as expected were concentrated at the tip of the needle where there is a great decrease in diameter. The shear rates that would be expected with increasing normal stress at the plunger range from $50 - 200 \text{ s}^{-1}$. All geometries printed were designed in Solidworks (Dassault Systèmes) and converted into an STL file. As described above these files were then imported into Slic3r to generate G-code instructions for the 3D printer and sent to the printer via Repertier Host. Printed line definition for controlled deposition of bioinks was evaluated with profilometry. The peak height and filament width were calculated as averages of three separate measurements across the entire length of the filament. The peak height's dependence on the layer height was not linear ranging from 21 to 270 μm for 1 to 5 layers respectively. The dependence of the peak height with increasing number of layers was fit to a second order polynomial with a fit coefficient of 0.987. This can be used to predict the layer height to be programmed into the slicing software. The filament width did not show significant variation across layer height ranging from 755 to 900 μm .

Once the fluid and viscoelastic properties of the bio ink were characterized, the bulk mechanical properties of the printed constructs were determined. The goal was to achieve properties that could match those of native soft tissue. For native soft tissues and organs, the Young's modulus ranges from 0.1 to 1000 KPa [44]–[47]. We evaluated bio ink formulations with increasing protein concentration in unconfined compression and the resulting stress was plotted against strain (Figure 4a). The elastic modulus, recovery and hysteresis were measured from these strain curves (Figure 4c,d). The viscoelastic properties were measured under dynamic mechanical loading. A strain sweep (data not shown) at a constant frequency of 1 Hz was performed to establish linear viscoelastic region (LVR). A single point dynamic measurement was performed at 1Hz at 1% strain which was within the LVR. These viscoelastic properties of the bulk materials, loss modulus, storage modulus, and complex modulus are summarized in Figure 4.

There was a significant toe region from 0 – 5% strain (Figure 4a). Following ASTM standards for characterization of hydrogels and compression testing[48]–[50] an offset was applied and the linear regression was fitted from 5 to 10% strain. The resulting elastic modulus values (Figure 4b) show that there was statistical significance between concentrations tested showing a linear trend of increase in moduli with increase in protein concentration. The loss, storage and complex modulus also showed an increasing linear trend with silk protein concentration indicating that the bulk material mechanical properties of the crosslinked printed material is dependent on the silk concentration and can be tuned to match a range of soft tissues properties.

To demonstrate applicability in patient specific implants DICOM images were extracted from the patient's CT scan. The cheek geometry was extracted from DICOM images using threshold segmentation and was reconstructed and optimized into a 3DP format using Mesh Lab (Figure 5a). The model was then imported into Repertier Host were parameters for

printing such as infill could be modified. The anatomically relevant, fully crosslinked (physically) printed construct (Figure 5b) is shown.

Each animal had 1 printed construct and 1 lyophilized printed construct implanted in lateral pockets. The bio ink formulation (1:1 10% w/v silk to 10% w/v gelatin and 25% /w glycerol to silk) was prepared and cylinders of 4 mm diameter 2 mm thick were printed in a sterile setting. These cylinders were stored hydrated at 4°C until implanted. The and non-porous printed constructs (Figure 6) showed progressive cell infiltration and degradation as the duration *in vivo* increase with the 3 month non-porous sample showed infiltration into the entire construct. The cellular infiltration in the lyophilized porous implants was faster and more significant with the construct being infiltrated in 1 month.

4. Discussion

A new bio ink was studied that was processed at room temperature and in aqueous conditions, supporting the 3D printing of soft tissue systems. The mechanical properties and degradation of the ink could be tuned according to component ratios and concentrations. The biocompatible silk blend as well as the non-toxic printing and post process curing makes the system useful for the incorporation of bioactive components for functionalization. The thermoreversible behavior of gelatin at physiological temperatures makes it an attractive material for 3DP. Unmodified gelatin is soluble in aqueous solutions and must be crosslinked either by chemical means or enzymatic methods. Crosslinked gelatin gels are not mechanically robust while gelatin physically crosslinked with silk generates bio ink formulations with robust mechanical properties and avoids any chemical or photo crosslinking. The shear thinning rheological behavior of silk [51] makes it useful for extrusion-based printing. A library of self-curing silk polyol blends were previously evaluated toward this goal [8]. Following these initial formulations, we concentrated on the addition of gelatin as a bulking agent for 3-dimensional bio printing.

Gelatin serves as a sacrificial material that enables structural support during printing. Uncrosslinked gelatin would normally be soluble. As in other studies [52] gelatin becomes physically entangled when blended with silk and then physically crosslinked. We found similar results where gelatin by itself was completely soluble when immersed in PBS at 37°C. The solubility of the material decreased with increasing silk concentration but only for those samples that were physically crosslinked (beta sheet structure) as confirmed by FTIR. This indicates that the gelatin was physically entangled within the beta sheet structures of the silk.

In prior studies we explored silk-glycerol mixtures related to solubility and crystallization of the silk [8], [35]. In these studies, we found similar results; incorporating silk into the formulations resulted in more insoluble, stable materials but post processing with glycerol immersion was needed to generate fully insoluble material. It was hypothesized that unlike a pure silk where glycerol can induce physical crosslinking in the solution state when blended with another polymer, here there could be phase separation that does not allow for crosslinking to occur and post processing immersion was needed. Visually we examined the diffusion based physical crosslinking of immersion in glycerol.

Dilution of glycerol to 500 mg/ml supported improved diffusion into the gels, where crystallization could be visually observed as the gels gradually turned opaque. Phase separation was visible in the 1:2 formulations as the crystallized silk concentrated at the edges of the construct leaving soluble gelatin in the middle. This phase separation, while not explored here could be exploited to generate constructs with programmed degradation by varying the silk to gelatin ratio within the printed structures. Post processing with methanol immersion had an immediate effect on the structures, turning them opaque, but resulted in some protein aggregates separating into solution. This was confirmed with a greater initial mass loss in the methanol treated samples than those post processed with glycerol.

In order to determine printing conditions for the inks the fluid properties under steady shear and the viscoelastic behavior with dynamic oscillatory rheometer were studied. The thermoreversible behavior of the bioinks was controlled by gelatin concentration. The 10% w/v silk: gelatin formulations had a melting temperature of 18°C while 15% w/v formulations had a melting temperature of 22.8°C. Above these temperatures Newtonian like behavior was seen as the viscosity decreased linearly with increasing temperature, while below these melting temperatures there was non-Newtonian behavior. Extrusion was best conducted at a temperature where the ink material has Newtonian behavior and deposition should be close to the melting temperature. To further characterize the fluid behavior of the bioinks and establish an empirical model which could be used for fluid simulation, assessments of steady state shear ramps at the melting temperature and at 37°C for each formulation was performed. Based on the flow curves there was a power law shear thinning at shear rates below 200s⁻¹[38]. For equation 4 in the form of viscosity against shear rate the coefficient n known as the power law index characterizes the shear thinning behavior of a fluid. This index range from 0 – 1 and is indicative of non-Newtonian behavior of the material. The bioink formulation tested had a power law index of 0.97 indicating that it has Newtonian like behavior at 37°C. The parameter m which was 1.14 for the bioink formulation tested is the consistency index which describes the consistency of the fluid [38]. These parameters depict the fluid behavior and were used as inputs to simulate laminar fluid flow through a syringe.

During extrusion the bio ink was expected to undergo a concentration of shear stress at the needle tip due to large decrease in cross diameter from syringe barrel (mm) to tapered needle (100 – 500 μ m). In order to understand these shear rates the fluid flow through a syringe with a 25 gage needle was assessed. The fluid model was solved for the maximum shear rate with increasing normal stress at the plunger. A shear rate of 100 – 200 s⁻¹ would be expected, consistent with similar studies done for other hydrogel based systems [53]. From the steady shear ramp data at these shear rates the material was stable in the Newtonian regime as described above.

The 10% w/v 1:1 silk to gelatin ratio was chosen for printing. This formulation allowed for printing at physiological temperatures (extrusion at 37°C and deposition around 20 to 25°C) and the structures were stable once 3D printed. Print resolution and accuracy experiments were also conducted using this formulation. Increasing layer height had a quadratic additive effect on the peak height which could be described by a second order polynomial. This would be expected as the layers were increased the bottom layers dried and were reduced in

height. This phenomenon could be accounted for in the print design and can be adjusted in the Gcode and layer height parameters. The filament width showed no statistically significant variation as layer height was increased. Typically, during printing the first layer has the most variation in filament width and height and due to cohesion of the biopolymer ink to the surface, with each added layer more consistent leading to cone like patterns (Figure 3).

Our goal was to generate a printed material with mechanical properties that would match native soft tissue. The mechanical properties of the scaffold materials could be tuned by increasing protein content, with elastic moduli from 50 Pa to 1000 Pa. These values are well within the range of soft tissues [44]–[47]. The viscoelastic properties were assessed to understand the stability of the bulk material. We found that the loss and storage modulus were independent of frequency indicating a stable solid structure. The storage and loss modulus of the bulk material was compared against the shear moduli obtained from oscillatory shear rheometer. In the fluid state we expected the moduli to be dominated by the thermoreversible behavior of gelatin, while in the solid state silk acts as the structural material and should dominate the moduli. This hypothesis was confirmed by our experimental data as increasing silk concentrations increased the storage modulus in the solid state but not in the fluid state. This result indicated that the silk in the system acts as the structural component while the gelatin acts as a supporting sacrificial material.

Autologous adipose tissue is the gold standard for reconstruction of oral and maxillofacial soft tissue defects. Autologous fat graft survival is an inherent clinical problem with typically 20–90% graft volume loss within months [54], [55]. These grafts carry complications such as the need for additional intervention and possible necrosis of the implanted tissue. Current approaches to overcome these shortcomings have been the use of biomaterial to act as a structural matrix to fill defects and induce regeneration. The biomaterials can provide the biochemical and biophysical cues for tissue regeneration and are known to induce little inflammation [26]. A step forward for the field is the use of established biomaterial platforms to create custom geometries [56], [57]. Printing a patient specific geometry with a biocompatible material that retains shape over time would address the limitations with current treatments and meet the requirements for soft tissue replacement. To demonstrate that physiologically relevant geometries can be accommodated with the new inks, CT-scans from a patient that had undergone tumor removal were used to generate a scaffold to fill in a large area of soft tissue. Using the left cheek as a template a 3D reconstruction of the area was segmented and converted into a printable model which was then printed with the ink described earlier. This physiologically relevant printed structure confirmed that our system would be useful for filling customized soft tissue defects.

In vivo evaluation of the 3D printed silk: gelatin: glycerol gels revealed that the dense non-porous structures printed had excellent shape retention. Cellular infiltration was initially observed at 2 weeks and more significantly by 4 weeks as the material started to degrade and was infiltrated by channels 2 mm into the middle of the 4 mm structure. At 3 months the gel structure was broken down and collagen was deposited. There was an absence of multinucleated giant cells at all time points indicating minimal tissue inflammation. Because the size of the implanted structures was limited to fit into a mice model, porosity could not

be programmed into the design. We however, showed (Figure 3) that we could generate various infill patterns with channels from 500 to 1 mm. These channels could help induce cellular infiltration as well and could be studied in larger sized devices for studies in large animal models. To introduce porosity into our constructs we lyophilized printed gels and evaluated the *in vivo* response to these implants. As anticipated, there was much greater cellular infiltration with the entire implant being integrated within 1 month. These lyophilized implants could be used in cases where greater infiltration is desired. The dense non porous implants would be suited for applications where shape and volume retention is needed.

5. Conclusions

Silk-based biopolymer blends were studied for 3DP for soft tissue implants. The system's rheology was suitable for 3DP and could be tuned to obtain different physical and mechanical properties suitable for soft tissue reconstruction. Silk-based bio ink formulations were optimized by modulating protein concentration and gelatin and glycerol concentrations to generate materials optimal under printing conditions. These formulations were designed to yield under extrusion forces, undergo rapid gelation upon deposition, and be biocompatible *in vivo* and show volume retention over time. We found that a gelatin concentration above 10% w/v was optimal for printing in physiological conditions (37°C extrusion and 20–25°C deposition) and a ratio of 1:1 of silk to gelatin was needed to maintain a homogenous stable structure. This bioink formulation was used to print patient specific cheek geometry that could be used to reconstruct soft tissue resected due to reconstructive surgical procedures such as tumor removal. The *in vivo* response of the printed implants was tested in a mouse model and shown to retain their shape up to 3 months with minimal inflammatory response and tissue integration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Mass of printed gel remaining with increasing silk to gelatin ratio after incubation up to 5 weeks. Silk : Gelatin : Glycerol in ratios of 2:1, 1:1, 1:2 were tested with two different post processing conditions glycerol bath immersion and methanol bath immersion. (B) Images of phase separation induced by varying silk to gelatin ratios, scale bars represent 1 mm. Four samples per condition were tested and the average mass remaining is shown with error bars indicating the standard deviation of the replicates. The full FTIR spectra (C) and the amide I region (D) of silk only and silk: glycerol: gelatin with three post processing conditions: no post process, glycerol bath immersion, methanol bath immersion. The spectra for silk only (a) shows an amorphous structure without the characteristic beta sheet peak while spectra for glycerol treated (b) and methanol treated (c) silk only samples show the appearance of this characteristic peak (indicated by vertical line). Similar results were seen for silk: gelatin: glycerol samples (d) no post treatment (e,f) glycerol and methanol treated all show this characteristic peak with the reduction of the amorphous peak.

Figure 2.

Temperature sweeps were performed from 0 to 40 $^{\circ}\text{C}$ at 1% strain and 1 Hz. The resulting complex viscosity was plotted against increasing temperature for increasing silk (A) and gelatin (B) concentrations. The melting temperatures were calculated as the point where there was an appreciable change in the complex viscosity and plotted for increasing silk (C) and increasing gelatin concentrations (D). (E) Summary of bio ink formulations tested. Increasing concentrations of silk (10% w/v to 15% w/v) and gelatin (10% to 15% w/v) were tested with a dynamic oscillatory rheometer.

Figure 3.
(A) Filament height and width with increasing layer height measured by interferometry. The peak height was fitted to a second order polynomial. (B) Printed cylindrical constructs with concentric pattern

Figure 4.

a) Stress plotted against strain for varying concentrations of a 1:1 ratio of silk and gelatin. b) Ramp down stress vs. strain up to 10% strain from which elastic modulus was calculated c) Recovery in height of gels strained to 40%. d) Hysteresis calculated as the ratio of the area under the curve of the stress strain load to unload cycle. e) Summary of bulk material properties under static loading, elastic modulus, and dynamic loading, storage and loss moduli.

Figure 5. Physiologically relevant geometry 3D printed. (A) CT scan of patient, segmentation of cheek geometry and reconstruction into a 3D printable model (B) As printed cheek geometry.

Figure 6.

In vivo interactions with implanted silk gels. Printed gels of 10% w/v silk and 10% gelatin w/v were implanted subcutaneously in a mouse model. Gels were explanted and examined histologically by a) H&E staining 2, b) 4, and c) 12 weeks following implantation. Scale bars are 500 μm .

Table 1

Bio ink formulations tested. Decreasing ratios of silk to gelatin were tested to determine stability of material under physiological conditions.

Bio ink	Silk % (w/v)	Gelatin % (w/v)	Glycerol %(w/w)
S-Gly:G 2:1	10	5	25
S-Gly:G 1:1	10	10	25
S-Gly:G 1:2	10	20	25

Table 2

Summary of bio ink formulations tested. Increasing concentrations of silk (10% w/v to 15% w/v) and gelatin (10% to 15% w/v) were tested with a dynamic oscillatory rheometer.

Bio ink	Silk % (w/v)	Gelatin % (w/v)	Glycerol % (W/W)
10S10G	10	10	25
10S13G	13	13	25
13S13G	13	13	25
15S13G	15	13	25
17S13G	17	15	25
10S15G	10	15	25