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Inkjet Printing of Regenerated Silk Fibroin: From Printable Forms to Printable Functions

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The development of varying approaches for biomaterials fabrication has been driven by the ability of these materials to operate at biological interfaces. Biomaterials have evolved from inert, monolithic matter, into bioinstructive, hybrid systems, with functionalities that are defined and designed as a function of desired target applications.^[1] This approach, however, does not foster multifunctional features for such material systems, as usually the end goal, such as mechanical matching to tissue or targeted degradation lifetime for the repair, results in more specialized biomaterial formats.^[2] Here, we investigate a different approach to biomaterials fabrication, developing a biomaterials approach where the system utility can be controllably reconfigured ab initio to enable multiple end uses. Using silk fibroin as the base biomaterial, a silk solution formulation was developed that can be doped with other components to generate

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a custom library of inkjet-printable, functional "silk inks" for use in sensing, therapeutics, and regenerative medicine. To illustrate this concept, different functional inks were generated and by the addition of nanoparticles, enzymes, antibiotics, growth factors, and antibodies during the printing process, with retention of functions.

The choice of silk protein as the major constituent for functional ink studies was due to aqueous solubility, non-toxicity, green chemistry, and polymorphic features.[3-5] Silk fibroin can be present in amorphous (random coil, water soluble) and in increasing levels of crystalline (beta-sheet, water insoluble) conformations, which allows solvation of the protein at high concentration (up to 20 wt%) or to modulate its stability in water.^[6] Polymorphism also enables silk-mediated stabilization of water-soluble, labile molecules, against heat and chemical (e.g., oxidative) stresses, incorporating them into the protein structure upon drying.^[7] Silk-mediated stabilization can be further enhanced when the amorphous structure of silk is converted into the crystalline state (e.g., through exposure of the material to water vapor under low vacuum at room temperature) upon the evaporation of water.^[8] Additionally, the amphiphilic nature of silk protein chains^[9,10] generates solution drops of silk in the pico- to nanoliter orders, enabling use in printing technologies without modifying the rheological properties of the inks (e.g., viscosity, surface tension) with non-compatible additives, such as organic or inorganic surfactants, that can negatively interact with the doping agents and alter the original physical properties of the inks (e.g., pH, ionic strength).^[11,12]

A drop-on-demand, piezoelectric-based inkjet printing process was utilized as the enabling method for controlled material deposition. Inkjet printing is a mature, ubiquitous technique. Piezoelectric-based inkjet technology generates droplets without the need for thermal actuation that can damage labile biological compounds contained within the inks.

Inkjet printing has recently drawn significant attention by extending applications to the life sciences (also known as bio-printing) thanks to widespread availability and ease of use.^[13,14]

The preparation and use of silk inks is a water-based process, eliminating the use of organic or inorganic solvents, thus supporting the addition of dopants and bioactive compounds, while providing compatibility with commercial inkjet printers and printer nozzles. The silk inks in this work were prepared by degumming (sericin removal) raw *Bombyx mori* silk fibers, followed by water-based extraction and purification (see the Supporting Information for details). The preparation of functional silk inks was carried out using an entirely aqueous system, without additives/surfactants, in ambient conditions of temperature and pressure.^[15] Generally, the most important





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Figure 1. Overview of preparation and inkjet printing of silk inks. a) Schematic of the preparation of silk inks – native silk fibers are dissolved to yield a solution of appropriate viscosity for printability through piezoelectric ink nozzles, as shown in the image (b) depicting silk droplets ejected from nozzles. Addition of compounds to this silk-ink formulation allows printing of functional inks. Examples of patterned functional silk inks are shown: c) silk ink containing gold nanoparticles (Au-NPs) printed on paper: d) silk ink containing HRP after exposure to TMB, e) silk ink with ampicillin printed on a bacterial lawn – the printed pattern illustrates the areas in which bacteria growth was inhibited, f) silk ink containing BMP-2 for bone development after exposure to staining to verify calcification. The staining reveals that calcification follows the printed pattern down to sub-100 µm feature size.

intrinsic physical properties determining printability of an ink are viscosity (η), density (ρ) surface tension (γ), and nozzle diameter (d).^[16–18] Using a modified form of the Navier–Stokes equation,^[19] these physical properties can be used to estimate the balance between the capillary force, inertial force, and viscous force for stable droplet formation as described by the dimensionless quantity $Z = (d\rho\gamma)^{\nu_2}/\eta$. To first order, a solution is theoretically printable for 1 < Z < 10.^[20–22] The rheological properties of the as-prepared silk inks can be tuned within the range of γ between 0.04 and 0.07 N m⁻¹, and between 3 and 300 mPa s for dynamic viscosity at room temperature by adjusting the molecular weight of the silk (via the silk purification process – temperature, time).^[23] The final concentration of silk in solution can also be used to tune the rheological properties of the ink.^[23]

A commercial inkjet printer with a minimum droplet volume of 1 pL (Dimatix DMP 2800 equipped with Dimatix Materials Cartridges, DMC-11610 21 µm nozzle diameter, Fujifilm, Santa Clara, CA, USA) was used and the printable silk solution used for most of the work reported here was from silk extracted for 120 min (which provides a protein with a molecular weight in the 90–35 kDa range). The silk solution was subsequently diluted to ≈3 wt% with deionized water to achieve a surface tension of 0.046 ± 2 N m⁻¹ and a dynamic viscosity of 3 mPa s, yielding a silk ink with a calculated *Z* of \approx 0.33. The silk solution was filtered through a nylon membrane with 0.2 µm pore size and then compounds/molecules were mixed into this solution to generate the functional silk inks. These inks were then loaded into printer cartridges. Drop formation and trajectory after ejection was monitored in real time using a drop imaging system and optimized by controlling the actuation profile of the piezoelectric nozzles including the number of nozzles in action, firing voltage and frequency, pulse duration, and cartridge temperature (see the Supporting Information for details). Though the smallest spot size and line width varied as a function of the particular silk ink used, 20 µm spots were reproducibly generated over tens of square centimeters (limited only by the printer used). Similar results with resolution down to $\approx 10 \ \mu m$ using silk inks were achieved with an electrohydrodynamic jet printing system (Figure S1, Supporting Information).

To illustrate the versatility of this approach, a small library of functional silk inks was prepared by adding nanoparticles, enzymes, antibiotics, growth factors, and antibodies. This enabled the ability to assess the compatibility of dopants with the silk inks and the inkjet printing process, as well as the suitability of the printed format for function (**Figure 1,2a**).

A silk ink containing Au-NPs was prepared and printed. Gold nanoparticles (Au-NPs) have been extensively used for



Figure 2. Functional silk inks. a) Schematic illustrating the preparation of three printed samples. b–d) Au-NP–silk inks: b) printed pattern of Au-NP–silk inks on paper. The paper is illuminated by green laser radiation inducing a topographical thermal distribution that follows the printed pattern. Increasing the incident laser power from $P = 12 \text{ mW cm}^{-2}$ (c) to 40 mW cm⁻² (d) caused an increase of $\approx 3 \,^{\circ}$ C in temperature (red zones in the thermal images). Multilayer printing can be applied to control the temperature differential of the printed inks (Figure S2, Supporting Information). e–g) HRP inks: (e) illustrates monitoring the activity of HRP silk inks at 1 d intervals showing the ability of the silk to preserve the activity of the enzyme. The word "bioinks" is printed on a paper substrate which is kept at 80 °C. f,g) Comparing the HRP printed alone (f) with the HRP printed in silk (g), activity was still detected through the colorimetric reaction after 18 h. h,i) Antibiotic–silk inks: h) different antibiotic concentrations were printed on a bacterial lawn – different clearing areas are shown in correspondence to the different printed concentrations. i) More complex patterns of printed antibiotic–silk inks can be obtained by regulating volumes and antibiotic concentration.

applications ranging from photonics (e.g., color engineering and surface plasmon resonance based sensing) to biology (e.g., bioimaging and hyperthermia therapy).^[24] This "plasmonic" silk ink was printed onto paper substrates and tested by exposing the printed silk to $\lambda = 532$ nm light (Figure 2b,d). Thermal images indicated a patterned distribution of heating that followed the profile of the printed pattern. Printing enables localization of plasmonic absorbers onto surfaces and tuning of the response as a function of the number of layers printed (Figure S2, Supporting Information). This approach can be extended to other inorganic dopants such as nanorods or quantum dots, and is attractive for the simplicity with which printed forms with dopant functions can be combined and easily designed.

One of the main appeals of using a water-based ink containing biopolymer molecules lies in the development of biologically active functional inks, by leveraging the ability of silk to entrain small functional biomolecules. Enzymes are widely used to develop functional materials, but are often hampered by a lack of long-term stability either during processing or in their final materials format. Using enzymes to generate functional silk inks is based on previous results on enzyme stabilization using silk biomaterials (particularly in bulk-loaded films and scaffolds) for medical, diagnostic, and biosensing applications.^[7] In this case, we used horseradish peroxidase (HRP) (Type VI, Sigma; 250–333 unit mg⁻¹) to assess the preservation of enzymatic activity in printed format (Figure S3a–c, Supporting Information).

The enzymatic activity of the HRP-doped silk solution before printing was evaluated and compared by a colorimetric enzyme-linked immunosorbent assay (ELISA) for horseradish peroxidase/3,3',5,5'-tetramethylbenzidine (HRP/ TMB) (Figure 2e–g). The HRP activities were measured by adding 100 μ L of TMB in 96-well plates containing 10 μ L of ADVANCED MATERIAL

HRP-containing solution for 1 min at room temperature and the reaction was stopped by the addition of 100 μ L of 0.1 $_{\rm M}$ sulfuric acid solution.^[25] Absorbance was measured at 450 nm using a commercial 96-well plate reader (BMG Labtech, Inc., Durham, NC, USA). HRP-doped silk ink solutions retained 82% of the initial enzymatic activity for up to 6 d, compared with 6% activity detected for HRP in water alone (i.e., without silk fibroin) when stored on the laboratory bench under ambient conditions (Figure 2e). The HRP-silk ink was loaded into cartridges and printed on conventional paper (Office Depot Brand Inkjet Print Paper). Little loss (<5%) of enzymatic activity was observed with 30 min of the printing process, compared with 40% loss for HRP printed in a water-based ink deprived of silk (Figure S4b, Supporting Information). Similar stabilization results for HRP during inkjet printing were observed previously;^[26] however, several additives such as glycerol, Triton, and sodium carboxymethyl cellulose were needed to adjust the rheological properties and to reduce ink evaporation. We have also seen the stabilizing impact of silk on HRP in solution in our prior studies.^[8] Additional testing was performed by storing the printed samples at room and elevated temperatures and checking activity over time (e.g., daily for 8 d at 25 °C -Figure S3d and S4c (Supporting Information) - and hourly for 8 h at 80 °C - Figure S3e and S4d, Supporting Information). The use of cellulose substrates preserved the enzyme functionality^[27,28] in the absence of silk up to 2 h when stored at 80 $^\circ$ C and for 2 d at 25 °C in our case (Figure S5, Supporting Information). The addition of silk extended the stability at both temperatures (Figure 2e,f and Figure S3-S5, Supporting Information).

The ability to biologically activate silk inks could prove useful to add therapeutic functionality to printed formats. For this purpose, silk inks were prepared by mixing antibiotic salts such as sodium ampicillin with the silk solution (Figure 2h,i). Previously, inkjet printing of antibiotics was explored as a novel mean of preventing the formation of biofilm colonies on orthopedic implant surfaces.^[29] Here, the use of silk fibroin enabled the stabilization of the antimicrobial agents, which was consistent with previous findings on antibiotics stabilization within silk materials in various formats.^[30,31] In particular, the stability of ampicillin incorporated into silk inks was higher than ampicillin stored in water after printing at various temperatures from 4 °C (refrigerator temperature) to 37 °C (body temperature) (Figure S6, Supporting Information). This combination of silk and antibiotic offers the ability to topographically control drug distribution, suggesting options for rapid antimicrobial assays that could identify the minimum inhibitory concentration and susceptibility evaluation of certain antibiotics (see the Supporting Information for details), or to design topographical therapy distributions to follow complex and/or extended wounds by printing a matching "therapy pattern." This concept is exemplified in Figure 2h,i to show the effects of a printed antibiotic pattern on a bacterial lawn. The clearing zone of the bacteria followed the pattern and was dependent on local content, which is controllable by the number of printed layers and spacing of the drops, along with the concentration of antibiotic in the silk ink (Figure S7, Supporting Information).

Topographical control can be also used for function related to directed tissue growth. Natural extracellular matrices



(ECMs) where cells are hosted are complex and variable in chemical composition and mechanical properties. Recently, there have been significant developments in the use of inkjet printing for cell and tissue engineering where living cells were directly printed in a spatially controlled manner. Bioprinting technology was used for cell guidance by defining surface patterns and concentration gradients with inkjet printed growth factors to control stem cell differentiation.^[11,32-36] In this work. fibroin protein films were used to confer mechanical stability and immobilize proteins to keep them viable for cell culture experiments, adding to the previously shown possibility of using inkjet printing to immobilize "solid-phase" gradients of growth factors to influence cell fate.^[37,38] The use of functionalized silk inks for bioprinting is particularly appealing given the potential to mediate cellular activities such as adhesion, migration, proliferation, and differentiation by programming/ defining a variety of structural and molecular (plus electrical and mechanical) cues in a spatial and temporal manner, while also providing prolonged stabilization of labile, bioinstructive (e.g., growth factors) by regulating their time-dependent release.

To test this concept, a functionalized Petri dish was developed to modulate human mesenchymal stem cell (h-MSC) fate by printing patterns of bone morphogenetic protein 2 (BMP-2) doped silk inks on the surface of the plastic dish (Figure 3). Once printed, the substrates were left at room temperature for 14 d before culturing h-MSCs in osteogenic media (deprived of any BMPs). Positive Alizarin red staining for cell-induced mineralization demonstrated the retention of stabilization of the heat-labile BMP-2 for cell utilization and the ability to pattern the mineralization outcome by following the printed design (Figure 3a,b). This result well corroborates the previously shown possibility of spatially controlling osteoblastic differentiation of C2C12 myogenic precursor within 24 h of bioprinting BMP-2 growth factors on microporous scaffolds.^[38] In addition, higher concentrations of BMP-2 in the silk matrix printed within the same Petri dish corresponded to increased levels of mineralization as shown by qualitative ATR-FTIR microscopy analysis and staining intensity, indicating microscale topographical control over mineralization as a function of the initial BMP-2 concentration in the silk ink (Figure 3c). Where BMP-2 growth factors were printed without silk fibroin, no positive Alizarin Red staining was visible.

The ease with which silk inks can be prepared in a waterbased process provides a wide set of opportunities by combining silk ink with other macromolecules such as antibodies, carbohydrates, lipids, nucleic acids, and functional polymers. This opportunity would provide useful avenues for printed functional forms such as for the design of sensors and assays. As a proof of concept, a colorimetric bacterial sensor was fabricated by directly printing functionalized silk inks onto various substrates (e.g., paper, transparent plastic sheets, surgical gloves). The inks were formed by immobilizing and stabilizing polydiacetylene (PDAs) vesicles conjugated with goat IgG antibody (ViroStat, #1094) for the detection of Escherichia coli in the printed silk materials (Figure 4a-c, Figure S8a-d, Supporting Information). Silk provided mechanical and biochemical stabilization functions for the labile antibody as the printing of the ink deprived of silk fibroin resulted in the deposition



Figure 3. BMP-2 ink for topographical control of h-MSC fate toward osteoblastic differentiation. BMP-2–silk ink with increasing concentration of morphogen printed on Petri dishes and left on a bench for 14 d at room temperature. Silk ink (without BMP-2) was used as control. a) Osteoblastic differentiation of h-MSCs cultured in osteogenic media (deprived of BMP) on the functionalized Petri dishes was assessed with Alizarin red staining for calcium at days 7, 14, and 21. Pictures of the stained Petri dishes showed that longer culture time and higher concentration of BMP-2 in the printed silk ink resulted in increased deposition of calcium phosphate by the cells. Scale bar = 4 cm. b) Optical microscopy images of Alizarin red stained Petri dishes, (a) depicting time- and BMP-2 concentration-dependent cell-induced mineralization. Increasing concentration of "red areas" for longer culture time and higher concentration of BMP-2 in the silk ink. Scale bar = 250 µm. Cell-induced mineral deposition investigated with ATR-FTIR microscopy. c) Representative ATR-FTIR spectra taken on printed zone of the functionalized Petri dishes after 21 d of h-MSC culture in osteogenic media. The higher concentration of BMP-2 in the silk ink corresponded to increased absorption of the $v_3 PO_4^{3-}$ peak centered at 1021 cm⁻¹, compatible with the cell-induced deposition of hydroxyapatite on the printed area. d) Measurement of the $v_3 PO_4^{3-}$ /Amide I absorbance ratio provided qualitative analysis of mineral deposition. The higher the ratio, the higher the mineral deposited by cells. Two-way analysis of variance (ANOVA) showed a time- and BMP-2 concentration-dependent increased by cells. Two-way analysis of variance (ANOVA) showed a time- and BMP-2 concentration-dependent increased by cells. Two-way analysis of variance (ANOVA) showed a time- and BMP-2 concentration-dependent increased by cells. Two-way analysis of variance (ANOVA) showed a time- and BMP-2 concentration-dependent increased by cells. Two-way analysis of variance (A

of material that was wearing out the substrate once exposed to bacterial broth. The sensing mechanism was based on the intrinsic color change of PDA (from blue to red) produced by a conformational change in the PDA under external stimuli (which results in a more disordered, less coplanar, polymer with decreased bond conjugation length, Figure S8e–g, Supporting Information).^[39–42]

Upon printing, the colorless DA–IgG–silk blend was exposed to UV (254 nm, 5 min) to induce polymerization of DA (PDA), which resulted in a blue color of the material, captured by a digital camera (Figure 4d,e). The printed PDA–IgG–silk blend was then used as a colorimetric bacterial sensor by exposure to *E. coli*. A color change from blue to red was observed (Figure 4f), when exposed to $\approx 10^2$ CFU mL⁻¹ of bacteria, induced by the conformational change (stretching) of the PDA molecules under external stimulus (e.g., exposure to *E. coli* bacteria). This type of sensor would be particularly attractive for the rapid in situ assessment of pathogenic contamination by providing the sensing elements to be in direct contact with surfaces of interest including the body, food, and clinical equipment. A proof-of-principle application^[43] was demonstrated by printing functional PDA–IgG–silk inks on laboratory gloves and illustrating color change of the printed pattern upon exposure to high levels of *E. coli* (~10⁴ CFU mL⁻¹) (Figure 4g,h and Figure S9, Supporting Information).

Biomaterial polymorphism enables the confluence of form and function applicable to commercial inkjet printing technology. Tuning the rheological properties of water-based silk solutions provides a common base material for applications in plasmonics, tissue engineering, and biosensing. Based on the use of this biologically derived protein from naturally available materials, water-based silk fibroin inks exhibit many attractive features including printer-friendly rheological properties, compatibility with other water-soluble molecules, controllable degradation profiles, superb mechanical properties, and the ability to make biochemical compounds available in printed formats. The properties of silk inks can be extended to cover device needs by the inclusion of dopants exhibiting enhanced absorption, or tissue-specific needs to influence cell responses. Mechanical (e.g., stiffness) and biochemical







Figure 4. Polydiacetylene/IgG/silk ink for colorimetric bacterial sensing. Polydiacetylenes (PDAs) vesicles (a) were conjugated with goat IgG antibody (b), which were immobilized and stabilized in silk solutions for printing (c). d–f) Photographs of as-printed PDA-IgG-silk hybrid inks on plastic substrates. The colorless DA–IgG–silk blend (d) was exposed to UV (254 nm, 5 min) to promote the polymerization of DA (PDA), which resulted in blue colorization (e) of the material. The printed PDA–IgG–silk blend was used as a colorimetric bacterial sensor by exposing it to *E. coli* and changing color from blue to red with a bacterial concentration of $\approx 10^2$ CFU mL⁻¹ (f). A proof-of-principle application was demonstrated on surgical gloves with printed patterns turning from blue (g) to red (h), showing "CONTAMINATED," after exposure to *E. coli* at a concentration of $\approx 10^4$ CFU mL⁻¹, indicating contamination.

(e.g., exposed amino acidic sequences) properties can be tuned by controlling silk polymorphism and by using biomaterial composites (e.g., the addition collagen, keratin, tropoelastin) for additional functions. Furthermore, a large variety of bioinstructive molecules (e.g., growth factors, cytokines, enzymes) can be doped into the silk inks and stabilized within the printed material to provide an effective method to investigate cell-material interactions at the microscale and to functionalize the surface of inert materials with micrometer resolution.

The growth of bioprinting provides a compelling research and development tool, and silk fibroin could play an important role as an ink material that can be readily functionalized to extend printed forms with extended and useful functions.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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