Intrinsically Colored and Luminescent Silk

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Silk has been a highly prized material since its discovery a few thousand years ago, with a current annual industrial output of approximately 30 billion US dollars in China alone.[1,2] In silk industry, the outer layer of silk (sericin) needs to be removed in order to use the core of silk (fibroin) that has excellent mechanical properties combined with luster, smoothness, and comfort. To impart color to the finished products, silk fibroin is subjected to the dyeing process including steps to remove excess dye molecules and to restore the properties of silk that are altered due to the harsh conditions involved in the process.[3] Here, we demonstrate an in vivo uptake of dyes into domesticated silkworms, leading to the direct production of intrinsically colored silk by the silkworms. The biological incorporation of dyes into silk fibroin is a greener method of producing colored silk because it eliminates the need for an external dyeing process, along with the resources (water, energy, additional chemicals) and post-treatments associated with it. A series of fluorescent dyes were successfully used as model compounds to investigate and understand their selective uptake into fibroin or sericin through fluorescence imaging and spectroscopic quantification. A better understanding of the molecular factors that determine the uptake of substances into silk fibroin was established to select and design appropriate molecules for producing intrinsically colored and luminescent silk fibroin, i.e., by controlling the structure-dependent hydrophobicity and self-assembly capability of these molecules. In addition to the production of intrinsically colored silk for textile applications, the current work also results in a biocompatible and luminescent silk scaffold that allows better visualization of cells and monitoring of the scaffold performance over time. When applied to other compounds with similar molecular properties, this process can potentially lead to functional silk for various biomedical applications including tissue engineering and bioelectronic, bio-optic, and biomicrofluidic devices.[4–6]

Experimentally, the first model compound, rhodamine B was mixed into mulberry powder at a concentration of 0.05 wt% to make modified feed that was then fed to silkworms starting from the third day of the fifth instar. The obvious color change of the silkworm body within 1 hour demonstrates an effective uptake of this dye in vivo (Figure S1A, Supporting Information). The silkworm started producing intrinsically colored silk on the tenth day of the fifth instar, forming a pink cocoon within a day (Figure S1A, Supporting Information). Lowering the concentration of rhodamine B (0.01 and 0.025 wt%) led to less intensely colored cocoons, while increasing the concentration (0.10 wt%) did not result in significant increase of color intensity, as shown in Figure S2 (Supporting Information). Aside from the color, no other physical difference was observed between the colored cocoons and the white cocoons that were produced by silkworms consuming normal non-modified feed (Figure S1B, Supporting Information). Both the silkworm body and the resulting silk are also highly luminescent, appearing orange under UV irradiation. This luminescence is used to analyze the obtained silk.

Upon reeling the colored cocoons, colored silk was obtained as shown in the photograph taken under room light (**Figure 1**A). In each strand of silk, two parallel fibroin filaments are held together by a gummy outer layer of sericin, as observed by scanning electron microscopy (SEM) in Figure 1B. Rhodamine B was dominantly distributed in the two fibroin filaments as revealed by confocal microscopy (Figure 1C). The dominant uptake of rhodamine B into fibroin instead of sericin is clearly seen from a darker region in between the two luminescent fibroins, which is occupied by sericin as illustrated in Figure 1D. To verify this observation, the sericin was completely removed through a degumming process using a protease enzyme (Savinase). The resulting degummed silk consist of individual fibroin filaments (Figure 1F) that were still strongly colored in room light (Figure 1E) and highly luminescent under laser irradiation (Figure 1G). The ≈80% retention of rhodamine B in fibroin after a complete removal of sericin (**Figure 2**E) confirmed the effective uptake of this dye into silk fibroin (Figure 1H), which is important because fibroin is the commercially valuable part of silk for textile and biomedical applications.

The feeding method was previously attempted to produce colored silk,[7] and genetic engineering was employed to produce luminescent silk.[8] In this work, we have shown for the first time that the coloration and luminescence in silk produced by silkworms on dye-modified diet is due to the uptake of dye molecules into fibroin, made possible by specific molecular properties of these dyes. The colored silk has been achieved by adding appropriate dyes into silkworm feed with minimal interference to standard sericulture procedure, thus making this method applicable for large-scale production of colored silk directly from silkworms. The intrinsically colored silk described here is also different from the naturally colored silks produced by wild silkworms (e.g., Thai golden silk produced by wild

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DOI: 10.1002/adma.201003860

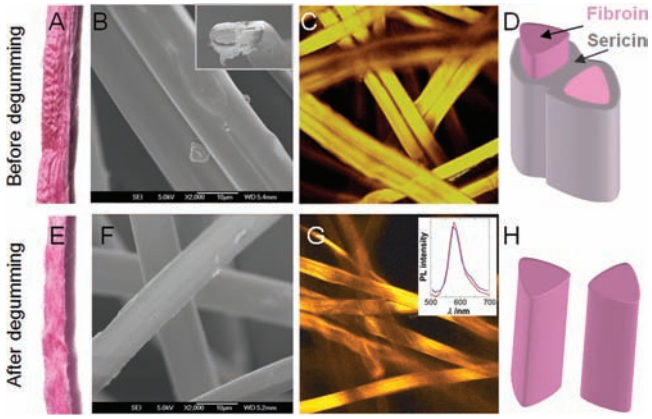

Figure 1. Selective uptake of rhodamine B into fibroin compared to sericin. A,E) Photographs of silk under room light before and after degumming. B,F) SEM microscopy images of silk before and after degumming. Inset in (B) is the cross section of silk. C,G) Confocal images at 40× magnification with 488-nm laser excitation before and after degumming. Inset in (G) is the emission spectra of the degummed silk, which closely matches that of rhodamine B in solution. D,H) Schematic diagrams of silk and degummed silk.

Saturniidae), in which the majority of the natural pigment from mulberry leaves are mainly accumulated in sericin rather than fibroin and the color was lost almost completely upon degumming. Confocal images of the Thai silk showed a luminescent sericin region with two dark fibroin filaments, as shown in Figure S3 (Supporting Information).

Besides rhodamine B, other fluorescent dyes can also be taken up in silkworms and further incorporated into silk for in vivo production of various colored cocoons. Among them, rhodamine 110 and rhodamine 101 were successfully taken up by silkworms to produce cocoons with various visible colors (Figure 2A). Under UV irradiation, these colored cocoons and fibers exhibited green, orange, and pink luminescence as opposed to blue for the cocoon produced by silkworm with normal feed (Figure 2B,C). The corresponding maximum emission wavelengths were at 518, 578, and 602 nm, respectively. More colors can also be readily achieved using the same method by mixing multiple dyes prior to feeding (e.g., rhodamine B + rhodamine 110).

Figure 2E summarizes the measured amounts of various fluorescent dyes (Figure S4, Supporting Information) that are taken up into silk and its components as a function of partition coefficient (log*P*, a measure of hydrophobicity). The amount of dye in the silk increases with the increase of log*P* up to ≈0.5, and then decreases with the further increase of log*P* close to ≈2.0 followed by a significant increase when log*P* is more than 2.0. At negative log*P*, only very low amounts of fluorescein sodium (log*P* = –0.79) and sulforhodamine 101 (–0.69) were found in silk because their higher hydrophilicity leads to a rapid clearance out of the silkworm's body. In comparison, the amount of rhodamine 116 taken up into silk was two magnitudes higher with a higher log*P* of 0.64. The uptake amount of rhodamine 116 in sericin was twice as high as that in fibroin; similar to the case of naturally colored Thai golden silk in which the natural golden pigment (0.55) primarily accumulated in sericin as mentioned earlier. With a further increase in log*P*, the uptake amount of rhodamine 110 (1.17) into silk started to decrease, and the uptake amount of acridine orange (1.80) into silk became very low. It was also found that more rhodamine 110 was taken up into sericin than fibroin.

At log*P* beyond 2.0, a sharp reversal of this trend was observed for rhodamine 101 (2.19) and rhodamine B (2.43) with more uptake into fibroin than sericin. The concentration of rhodamine B in fibroin was ≈350 ppm, which was 8 times, 16 times, and two magnitudes higher than rhodamine 116, rhodamine 110, and acridine orange, respectively, and 3 times higher than the amount of rhodamine B in sericin. This indicates the presence of another factor that affects the uptake and distribution of substances in silk, aside from molecular hydrophobicity. The dominant distribution of rhodamine B was studied by quantifying the dye content in the intrinsically luminescent silk secreted by silkworms. The concentration of rhodamine B in the silk gland just before the start of silk spinning reached ≈1 mM, as calculated from its concentration of ≈350 ppm in silk fibroin (Figure 2E). At this high concentration, dimers would be formed (Figure S5, Supporting Information).[9] Molecular self-assembly may expose either hydrophilic carboxylic acid or hydrophobic ethyl groups outward to change the hydrophobicity of rhodamine B dimers, leading to a more efficient transfer of the dye molecules into silk gland and eventually resulting in highly luminescent silk. In comparison, the concentration of

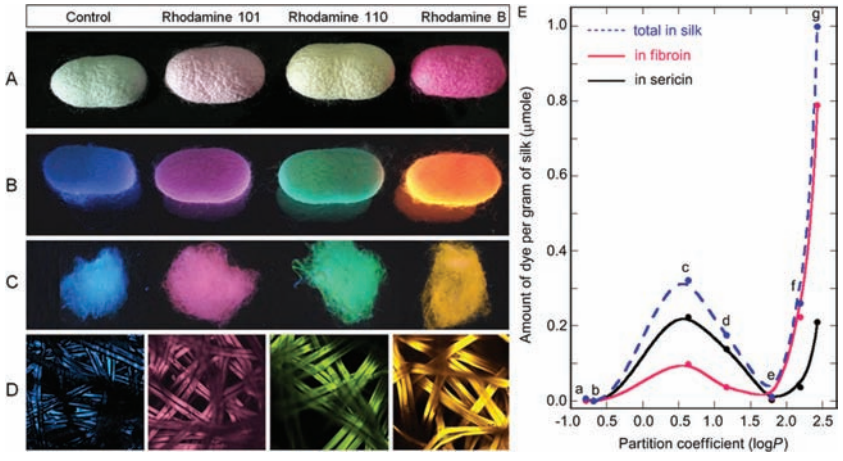

Figure 2. Intrinsically multicolored and luminescent silk produced by silkworms that have consumed various fluorescent dyes including rhodamine 101, rhodamine 110, and rhodamine B as compared to a control cocoon produced by a silkworm with normal feed. A) Photographs of colored cocoons under room light. B) Photographs of luminescent cocoons under UV irradiation. C) Photographs of silk fibers under UV irradiation. D) Confocal images of a small area of the colored cocoons under 488-nm laser irradiation. E) Quantification of various fluorescent dyes in sericin, fibroin, and silk as a function of their partition coefficient (log*P*). The curves plotted here are to show the correlation between hydrophobicity and uptake amount and are not representative of a model or equation. a) Fluorescein sodium, b) sulforhodamine 101, c) rhodamine 116, d) rhodamine 110, e) acridine orange, f) rhodamine 101, and g) rhodamine B.

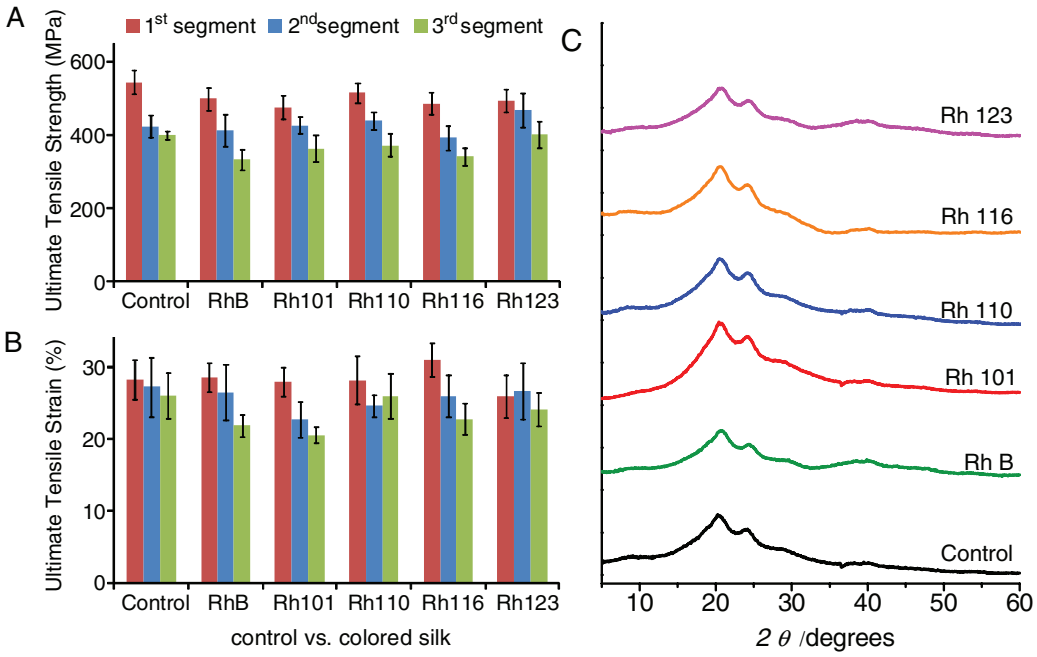

Figure 3. Mechanical properties of intrinsically colored silk compared to white silk (control). A) Ultimate tensile stress of various colored silk vs control. B) Ultimate tensile strain of various colored silk vs control. Each cocoon sample was divided into three 200-m segments during reeling, representing the outer, middle, and inner layer of the cocoons. (C) XRD patterns of various colored silk vs control.

acridine orange in silk was very low. This is because the non-amphiphilic structure of acridine orange does not allow tuning of hydrophobicity upon the formation of dimers.[10] Acridine orange molecules were unable to penetrate through hydrophilic sericin and were thus retained in silk gland cells.

The mechanical properties of these intrinsically colored silk were tested. **Figure 3**A,B show the tensile strengths and strains of various colored silk compared to white silk produced by silkworms with normal feed. Average tensile strengths and strains of colored silk range from 406–454 MPa and 23.7–26.5% respectively, which are comparable to that of white silk (≈455 MPa and ≈27.1%). The addition of fluorescent dyes, even at concentrations as high as 350 ppm, did not affect the crystallinity of silk and thereby preserved the mechanical properties of natural silk. This was verified by X-ray diffraction (XRD) data (Figure 3C), which show that the crystal structure and crystallinity of the colored silk are similar to that of white silk.[11] The mechanical values are not as high as those obtained for spider silk,[12,13] which could be due to differences in the supramolecular organization of the most basic units in silkworm and spider silks.[14–16]

Coupling excellent mechanical properties with high biocompatibility, white silk has been widely studied as a scaffold in tissue engineering, particularly where high mechanical loads or tensile forces are applied or where low rates of degradation are desirable.[17,18] In this research, a silk scaffold was constructed by forming a grid-like pattern on a glass cover slip using intrinsically luminescent silk containing rhodamine B. After degumming and sterilizing, human colon fibroblast cells (CCD-112CoN) were seeded and grown on the silk fibroin scaffold. After ten days, the cells were stained with fluorescein diacetate to show normal growth of the cells on the fibroin scaffold (**Figure 4**). The use of the luminescent silk as a scaffold allows an improved visualization of cells, whereby cells that are growing on or in direct contact with the scaffold appear yellow (due to the overlap of the green fluorescence of the fluorescein diacetate stain and the red fluorescence of the scaffold) while the other cells appear green. The clear interface between scaffold and cells also allows convenient monitoring of scaffold degradation. Overall, the scaffold made of intrinsically luminescent silk is not only biocompatible but also allows a better visualization of the performance of the scaffold. Other cell lines such as lung cancer cells (A549) were also tested with similar results.

In summary, a green method to produce a new class of functional silk and a better understanding of the effect of molecular properties on the biological incorporation of various molecular materials into silk fibroin have been developed. It was shown, for the first time, that the coloration in silk from silkworms fed on a dye-containing diet is due to the uptake of dye in the fibroin, as demonstrated through detailed characterization techniques. Aside from providing a cost-effective and environmentally friendly method for dyeing silk, which will be

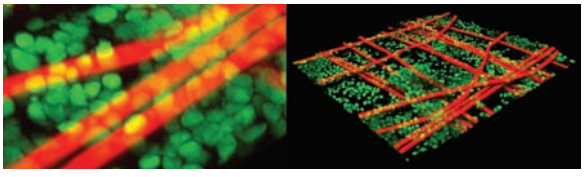

Figure 4. 3D confocal image of human colon fibroblast cells stained with fluorescein diacetate after culturing for ten days on a silk fibroin scaffold that was made of intrinsically luminescent silk containing rhodamine B.

valuable in the commercial silk textile industry, this work may also lead to a large-scale production of novel biomaterials with added functionalities. We can foresee a great demand to produce functional silk materials containing stimuli-sensitive dyes and various drugs that can be applied for wound dressing with monitoring or sensing features, tissue engineering scaffolds with antibacterial, anticoagulant, or anti-inflammatory features, and many others.

Experimental Section

Production of Colored and Luminescent Silk: Normal feed was a thick green paste prepared by microwaving mulberry powder (100 g, Recorp Inc., Canada) with of water (300 mL). Modified feed was prepared by thoroughly mixing an individual dye (50 mg) into normal feed (100 g). Domesticated silkworms (*Bombyx mori* larvae, Liang Guang II strain from China) were cultured up to the second day of the fifth instar with normal feed. They were then transitioned to modified feeds containing various fluorescent dyes from the third day of the fifth instar until complete spinning of their cocoons. These colored cocoons were then reeled to obtain silk for further processing.

Degumming of Silk: Typically, silk (100 mg) was added into a degumming solution (10 mL) consisting of Savinase enzyme (10 mg, Novozymes, Denmark) and Triton-X (10 mg) in 1 g L^{-1} $NaHCO_3$ solution pH 8, followed by heating at 55 °C for 1 h. The degummed silk was then collected and rinsed with water.

Quantification of Dyes in Silk: A piece of silk cocoon was weighed and degummed as described above. The resultant degumming solution was measured fluorescently to determine the amount of dye in sericin. The degummed silk was thoroughly rinsed and further dissolved by heating in 9 M LiBr solution at 70 °C for 3 h. Upon dissolution of the degummed silk, dye molecules were released and the obtained solution was measured fluorescently to determine the amount of dye in fibroin.

Cell Culture: Human colon fibroblast cells (CCD-112CoN) were obtained from American Type Culture Collection (ATTC, USA). Grid-patterned scaffolds were constructed by winding silk filaments around a glass cover slip followed by degumming and sterilizing. After seeding with $\approx 7 \times 10^5$ cells on each scaffold, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum for 8 d. The cells were imaged after incubating in 3.0-mL PBS (pH 7.4) containing 10 μg mL^{-1} fluorescein diacetate (95%, Sigma-Aldrich) for 5 min.

Supporting Information

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

Acknowledgements

This work was supported by the Institute of Materials Research and Engineering and the National University of Singapore

Received: October 19, 2010
Revised: January 12, 2011
Published online:

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